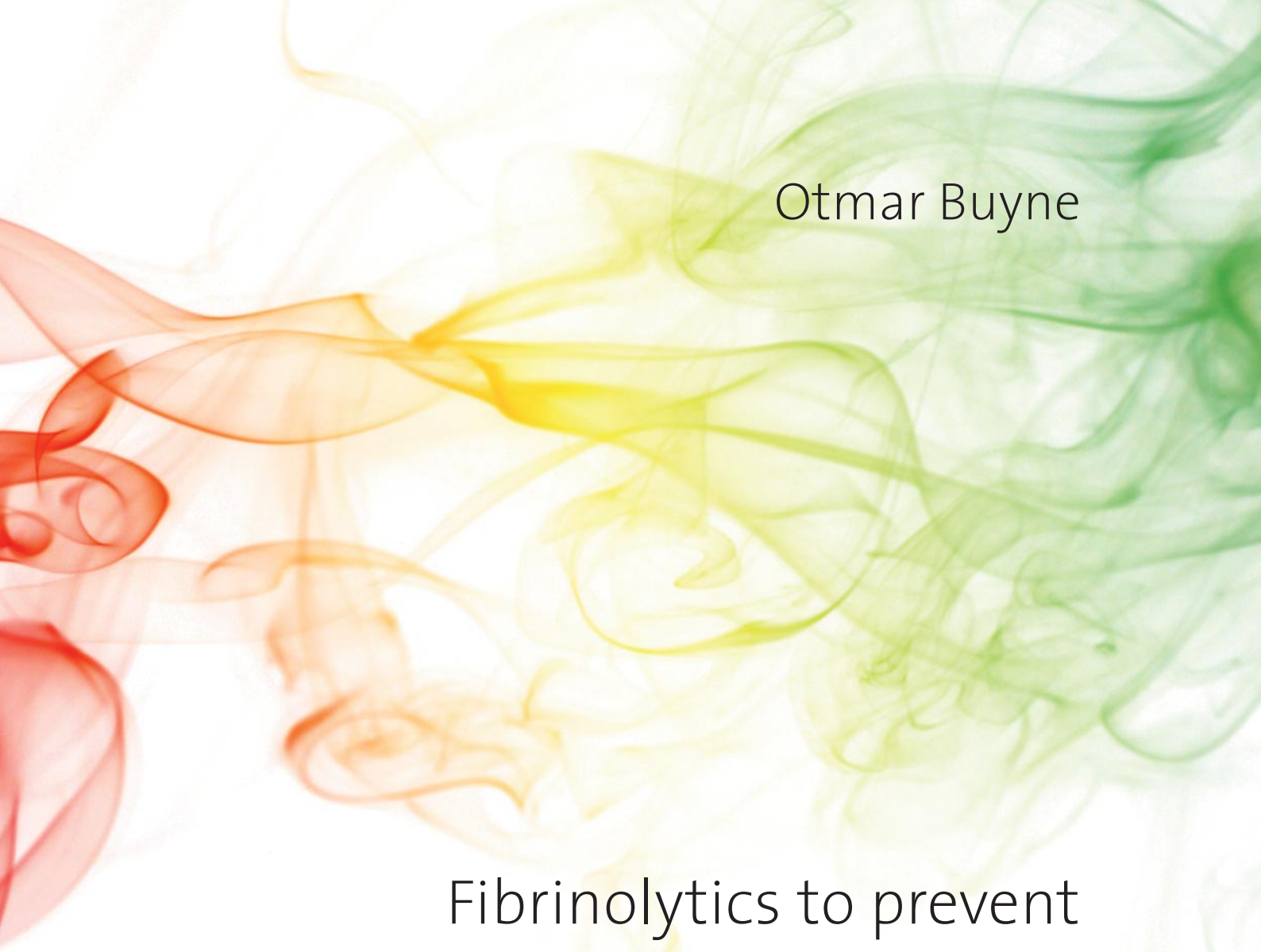

Otmar Buyne

Fibrinolytics to prevent intra-abdominal abscess formation in peritonitis

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Otmar Buyne

COLOFON

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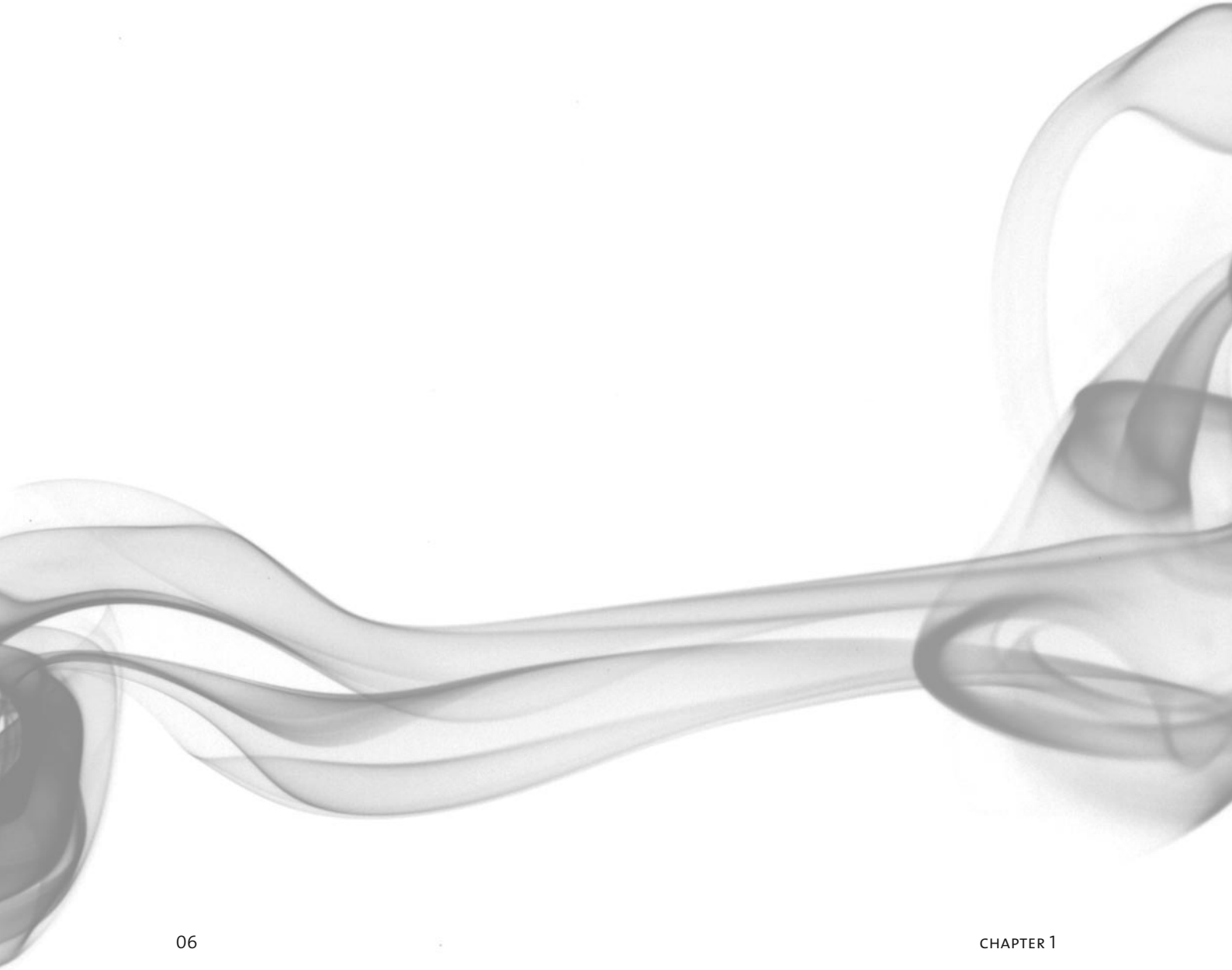


Chapter 1
Introduction

1. PHYSIOLOGIC DEFENSE MECHANISMS IN PERITONITIS

Intra-abdominal sepsis is a life threatening condition caused by perforation of the digestive tract, in the majority of cases. Contamination of the abdominal cavity with microorganisms will activate a chain of defense mechanisms to control infection. Bacteria that contaminate the peritoneal cavity are transported to the subdiaphragmatic spaces by the cephalad directed fluid stream in the peritoneal cavity. Via stomata in the mesothelial layer that covers the diaphragm, fluid and (bacterial) particles drain into the lymphatic lacunae of the diaphragm. The lymph is actively transported by the diaphragmatic muscle pump into the thoracic duct that drains into the subclavian vein(1-3). The microorganisms that enter the circulation evoke a systemic response and are eliminated by the immune defenses of the host(4).

Contamination of the abdominal cavity will elicit a local immune response. Upon contact with microorganisms, the mesothelium cells express a cascade of cytokines, chemokines and adhesion molecules, resulting in recruitment of polymorph nuclear neutrophils (PMNs) and macrophages to the peritoneal cavity. This process is partly mediated by peritoneal macrophages(5;6). The release of these cytokines and chemokines leads to mast cell activation, which in turn will release vasoactive mediators(7). These mediators increase vascular permeability, which is a general step in any inflammatory response and is aimed at carrying fluid, proteins and cells to the focus of infection. As a result a fibrin-rich exudate is formed containing cytokines and chemokines, which modulate the inflammatory response(8). In addition, the peritoneal exudate enhances opsonization(7;9;10).

In response to infection of the abdominal cavity, both coagulation and fibrinolysis are upregulated. In the early phase coagulation predominates, leading to the formation of fibrinous adhesions. Fibrin is of importance in preventing bacterial spread thereby reducing the systemic inflammatory response. In addition, bacteria can be trapped in fibrin clots, leading to containment of the infectious focus(11;12).

Normally, during the course of peritonitis, fibrin is gradually degraded by plasminogen activators (PAs). Tissue type plasminogen activator (t-PA) is the most important PA in peritonitis. T-PA is produced in the abdominal cavity(13), with mesothelium cells being the most important source(14;15;16). During resolution of inflammation, fibrinolytic activity will be upregulated. Upon fibrin degradation bacteria will gradually be released into the abdominal cavity and eliminated by the local immunological defenses.

Different factors have been identified that interfere with physiologic resolution of peritonitis.

1. The fibrinous adhesions that are involved in compartmentalization of infection can impair the circulation of abdominal fluid. In addition, fibrin may occlude the diaphragmatic stomata(17;18) thereby hampering the physiologic clearance of bacteria from the abdominal cavity. This will promote intra-abdominal abcess formation and adhesion formation; the former being a well-known source of ongoing or recurrent abdominal infection.
2. Bacterial synergy is known to adversely influence the outcome of peritonitis(19). Synergy is a complex phenomenon probably occurring on the basis of reduced peritoneal clearance as well as by bacteria providing reciprocal growth factors(20). In addition it has been reported that some microorganisms, for instance Enterococcus faecalis, not only contribute to bacterial synergy, but also may have a direct inhibitory effect on phagocytosis and intracellular killing(21).
3. Adjuvant substances such as blood or enteric juices adversely influence the local defenses. Bile for instance is associated with reduced peritoneal absorption and impaired local defense(22). Hemoglobin interferes with chemotaxis, phagocytosis, and phagocytic killing(23).
4. Like most pathogens, intraperitoneal bacteria adhere to peritoneal mesothelial cells and invade the submesothelial tissues(24).

If the local defense mechanisms in the peritoneal cavity fail, the inflammatory process in the abdominal cavity may become self-perpetuating which ultimately leads to the development of multi organ failure and death(25;26).

2. FIBRIN: FORMATION AND DEGRADATION

Fibrin

Fibrin is an insoluble, endogenous polymer, formed as the result of conversion of soluble fibrinogen by thrombin(14;27). Fibrinogen is a protein of 340,000 Dalton(28) mainly synthesized in the liver. The serum concentration varies with a half life-time in the circulation of approximately 3 days. Fibrinogen is a dimer, which consists of three peptide chains abbreviated with alpha, beta, and gamma(29).

Fibrin formation

The formation of fibrin is a well studied part of the intravascular coagulation cascade. The intra-abdominal coagulatory system has not been described in detail but probably occurs similar to intravascular clot formation (see figure 1). Classically, an intrinsic and an extrinsic pathway are described but in recent literature this discrimination is omitted since coagulation is supposed to be exclusively generated by Tissue Factor (TF). Thrombin formation at extravascular sites is initiated by expression of cell-anchored TF and propagated through assembly of the prothrombinase complex (30), via activation of factor VII, X and V, respectively. The prothrombinase complex catalyses the conversion of prothrombin into thrombin. Thrombin in turn is responsible for conversion of plasma fibrinogen into fibrin. The TF- factor Va complex can also activate factor IX, forming a tenase complex with activated factor IX and X, generating additional factor Xa, which amplifies coagulation. This is markedly facilitated in the presence of a phospholipids surface. Probably this surface ideally presented by platelets, is (partly) lacking in the abdominal cavity.

Tissue factor is present in plasma as are all other necessary clotting factors (31), but in peritonitis TF is expressed by mesothelial cells (30) and local inflammatory cells such as neutrophils and monocytes (32). The expression of TF is modulated by several inflammatory mediators and lipopolysaccharide (32;33).

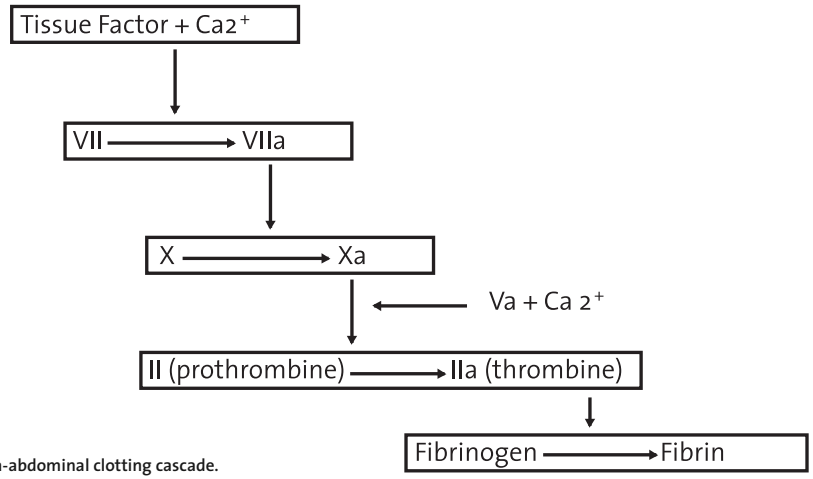


Figure 1. **Simplified presentation of the intra-abdominal clotting cascade.**

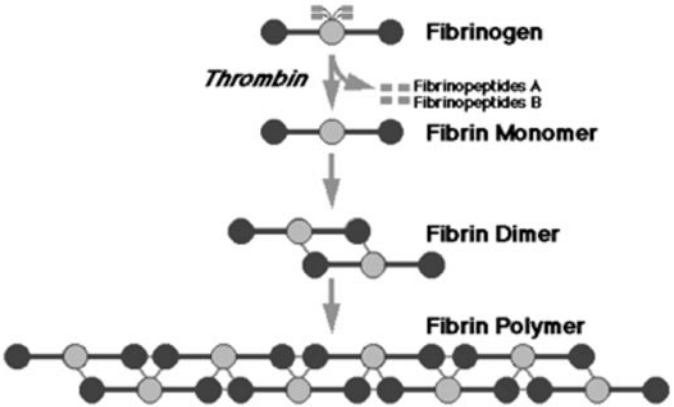


Figure 1. **The formation of fibrin.**
(By Douglas M. Tollefsen, Washington University, dept. of Hematology, with permission)

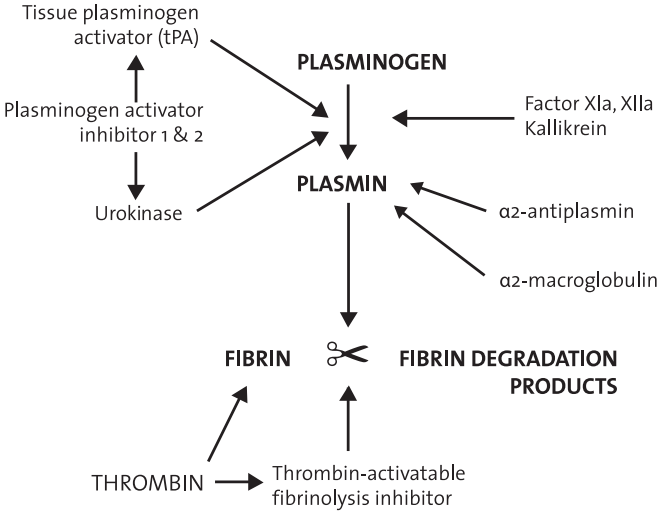


Figure 3. **Fibrin degradation.**

Cytokines and other inflammatory mediators are expressed when bacteria adhere to mesothelial cells. The inflammatory mediators induce mast cell degranulation and recruite inflammatory cells such as macrophages and polymorphonuclear neutrophils (PMNs) (34-37). In addition, vascular permeability increases in order to carry fluid, proteins and cells to the site of damage(7). Subsequently, a fibrinogen-rich exudate, that also contains clotting factors, is formed: the first step in fibrin formation in peritonitis(9;10;38).
Next, the dimeric fibrinogen molecule is cleaved by thrombin to produce soluble fibrin monomers, fibrinopeptide A and fibrinopeptide B. Fibrinopeptide A is frequently used as an early marker of fibrinogen-to-fibrin conversion(39). The plasma-borne enzyme thrombin not only contributes directly to coagulation by catalysing the formation of fibrin, but also orchestrates cellular effects such as platelet activation and vasodilatation(40).
Finally, enhanced by the thrombin-activated plasma factor XIIIa, the fibrin monomers are then cross-linked in the longitudinal as well as in transverse direction (Figure 2). After polymerization, a sticky fibrin network is formed, which explains the tendency of damaged or inflamed peritoneal surfaces to adhere to each other(27). As vascular patency will be restored after thrombus lysis, release of the attachment of adhesive peritoneal structures is achieved by lysis of the fibrin deposits(41). However, if the process of fibrin formation is unopposed by physiologic fibrinolysis, the fibrin mesh will become infiltrated by fibrocollagenous tissue thus forming fibrous adhesions(42).

Fibrin degradation

Under physiologic circumstances fibrin is degraded by an enzymatic system. This fibrinolytic system comprises a pro-enzyme plasminogen, which is converted into its active form, plasmin, by plasminogen activators (PAs). Plasmin in turn catalyzes the breakdown of fibrin into fibrinogen degradation products (Figure 3).
Two PAs have been identified: tissue type plasminogen activator (t-PA) and urokinase type plasminogen activator (u-PA). Both PAs are equally effective in lysing fibrin clots, but t-PA is much more fibrin specific and therefore less likely to act on systemic fibrinolysis (14;43); it has been detected in several types of tissue (44-46). In secondary peritonitis t-PA is produced in the abdominal cavity (13), with mesothelium cells being the most predominant source of t-PA production (14;15;15;16;47). Human peritoneal mesothelial cells (HMCs) play a critical role in maintaining the intraperitoneal balance between fibrinolysis and coagulation by expressing the fibrinolytic enzyme tissue-type plasminogen

activator (t-PA) as well as a specific plasminogen activator inhibitor, PAI-1, and the procoagulant protein tissue factor (TF) (47;48).

Altogether, fibrin is an important component of the fibrinolytic/coagulatory system in the inflammatory response in the peritoneal cavity. The wall of any abscess consists mainly of fibrin. Abscess formation on one hand leads to entrapment of bacteria, resulting in containment of infection, thereby reducing systemic spread of bacteria (49-51). On the other hand, bacteria embedded in an abscess are shielded from antibiotic therapy and from immunologic defense mechanisms (52-54).

3. AIM OF THIS DISSERTATION

This thesis reports the results of a series of experiments on the subject of reducing intra-abdominal abscess formation by intraperitoneal application of recombinant tissue type plasminogen activator. The following issues are addressed:

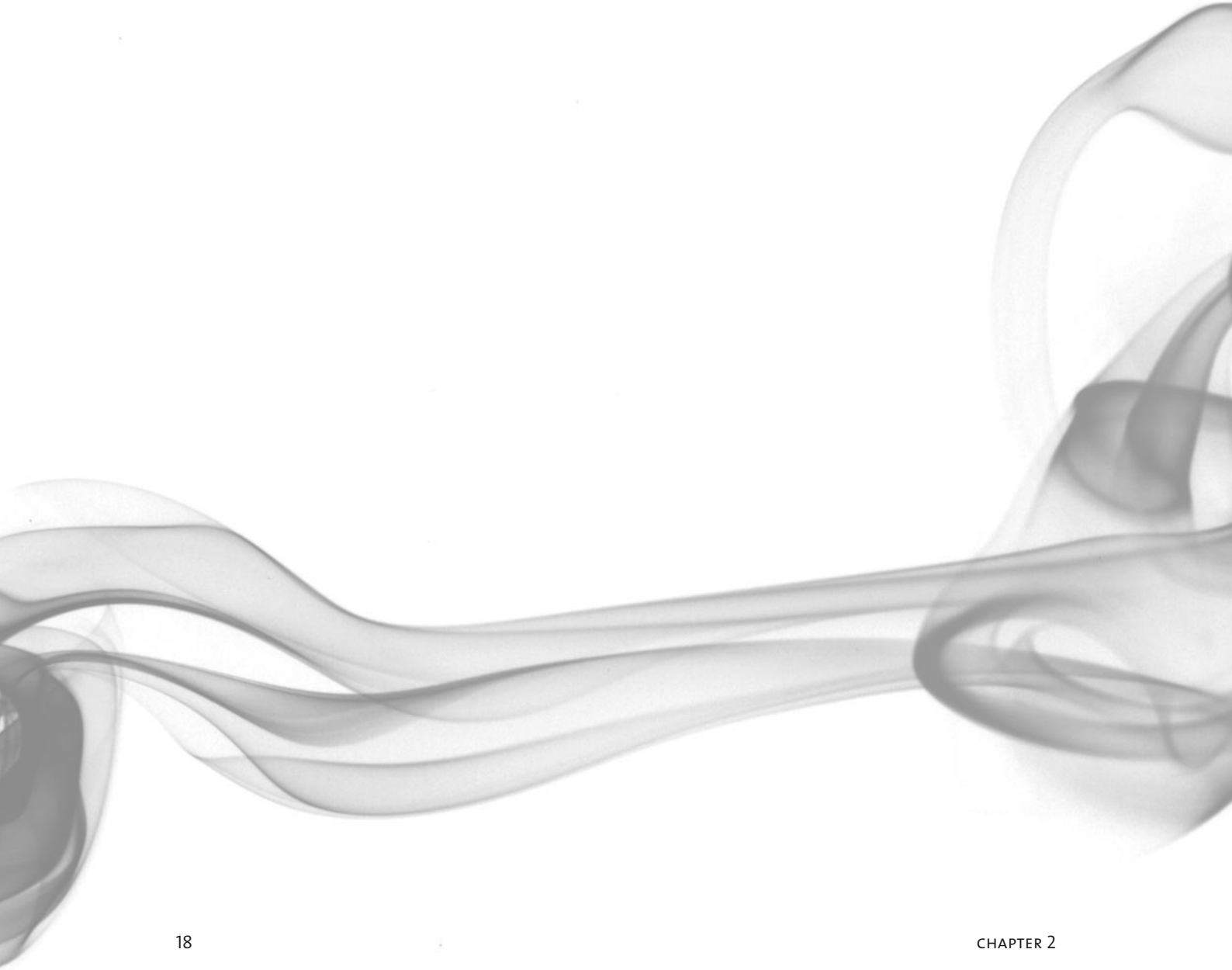
1. The development of a model, which allows for the study of mechanisms involved in the prevention of intra-abdominal abscess formation in generalized peritonitis.
2. To determine whether optimal abscess prevention can be achieved by changing the method of administration and to optimize the dosage scheme of intraperitoneally applied rtPA.
3. To investigate if both rtPA and urokinase can be used intraperitoneally to reduce abscess formation without inducing adverse side-effects.
4. To determine if prolonged intraperitoneal rtPA treatment is more effective than treatment during 24 hours and whether delayed rtPA treatment is still effective in reducing abscess formation.
5. To study the effects of systemic antibiotics on intra-abdominal abscess formation and survival, both in the absence and presence of intraperitoneally applied rtPA.
6. To study whether intraperitoneal application of rtPA impairs healing of bowel anastomoses and abdominal wall fascia after surgical debridement of secondary peritonitis in the rat.

REFERENCE LIST

(1) Michailova KN. Postinflammatory changes of the diaphragmatic stomata. Ann Anat 2001 Jul;183(4):309-17.
(2) Tsilibary EC, Wissig SL. Absorption from the peritoneal cavity: SEM study of the mesothelium covering the peritoneal surface of the muscular portion of the diaphragm. Am J Anat 1977 May;149(1):127-33.
(3) Tsilibary EC, Wissig SL. Lymphatic absorption from the peritoneal cavity: regulation of patency of mesothelial stomata. Microvasc Res 1983 Jan;25(1):22-39.
(4) Annane D, Bellissant E, Cavaillon JM. Septic shock. Lancet 2005 Jan 1;365(9453):63-78.
(5) Holzheimer RG, Schein M, Wittmann DH. Inflammatory response in peritoneal exudate and plasma of patients undergoing planned relaparotomy for severe secondary peritonitis. Arch Surg 1995 Dec;130(12):1314-9.
(6) Laroche M, Harding G. Primary and secondary peritonitis: an update. Eur J Clin Microbiol Infect Dis 1998 Aug;17(8):542-50.
(7) Kolaczkowska E. Shedding light on vascular permeability during peritonitis: role of mast cell histamine versus macrophage cysteinyl leukotrienes. Inflamm Res 2002 Nov;51(11):519-21.
(8) Farthmann EH, Schoffel U. Epidemiology and pathophysiology of intraabdominal infections (IAI). Infection 1998 Sep;26(5):329-34.
(9) Ellis H, Harrison W, Hugh TB. The healing of peritoneum under normal and pathological conditions. Br J Surg 1965 Jun;52:471-6.
(10) Hau T, Payne WD, Simmons RL. Fibrinolytic activity of the peritoneum during experimental peritonitis. Surg Gynecol Obstet 1979 Mar;148(3):415-8.
(11) Qiu G, Gribbin E, Harrison K, Sinha N, Yin K. Inhibition of gamma interferon decreases bacterial load in peritonitis by accelerating peritoneal fibrin deposition and tissue repair. Infect Immun 2003 May;71(5):2766-74.
(12) Reijnen MM, Bleichrodt RP, van Goor H. Pathophysiology of intra-abdominal adhesion and abscess formation, and the effect of hyaluronan. Br J Surg 2003 May;90(5):533-41.
(13) de Boer AW, Levi M, Reddingius RE, Willems JL, van den BS, Schroder CH, et al. Intraperitoneal hypercoagulation and hypofibrinolysis is present in childhood peritonitis. Pediatr Nephrol 1999 May;13(4):284-7.
(14) Tietze L, Elbrecht A, Schauerte C, Klosterhalfen B, Amo-Takyi B, Gehlen J, et al. Modulation of pro- and antifibrinolytic properties of human peritoneal mesothelial cells by transforming growth factor beta1 (TGF-beta1), tumor necrosis factor alpha (TNF-alpha) and interleukin 1beta (IL-1beta). Thromb Haemost 1998 Feb;79(2):362-70.
(15) van Hinsbergh VW, Kooistra T, Scheffer MA, Hajo van Bockel J, van Muijen GN. Characterization and fibrinolytic properties of human omental tissue mesothelial cells. Comparison with endothelial cells. Blood 1990 Apr 1;75(7):1490-7.
(16) Vipond MN, Whawell SA, Thompson JN, Dudley HA. Peritoneal fibrinolytic activity and intra-abdominal adhesions. Lancet 1990 May 12;335(8698):1120-2.
(17) Nagy JA, Meyers MS, Masse EM, Herzberg KT, Dvorak HF. Pathogenesis of ascites tumor growth: fibrinogen influx and fibrin accumulation in tissues lining the peritoneal cavity. Cancer Res 1995 Jan 15;55(2):369-75.

(18) Nagy JA, Morgan ES, Herzberg KT, Manseau EJ, Dvorak AM, Dvorak HF. Pathogenesis of ascites tumor growth: angiogenesis, vascular remodeling, and stroma formation in the peritoneal lining. Cancer Res 1995 Jan 15;55(2):376-85.
(19) Rotstein OD, Kao J, Houston K. Reciprocal synergy between Escherichia coli and Bacteroides fragilis in an intra-abdominal infection model. J Med Microbiol 1989 Aug;29(4):269-76.
(20) Dunn DL, Barke RA, Ewald DC, Simmons RL. Effects of Escherichia coli and Bacteroides fragilis on peritoneal host defenses. Infect Immun 1985 May;48(2):287-91.
(21) Montravers P, Mohler J, Saint JL, Carbon C. Evidence of the proinflammatory role of Enterococcus faecalis in polymicrobial peritonitis in rats. Infect Immun 1997 Jan;65(1):144-9.
(22) Andersson R, Schalen C, Tranberg KG. Effect of bile on growth, peritoneal absorption, and blood clearance of Escherichia coli in E coli peritonitis. Arch Surg 1991 Jun;126(6):773-7.
(23) Heemken R, Gandawidjaja L, Hau T. Peritonitis: pathophysiology and local defense mechanisms. Hepatogastroenterology 1997 Jul;44(16):927-36.
(24) Yao V, Platell C, Hall JC. Role of peritoneal mesothelial cells in peritonitis. Br J Surg 2003 Oct;90(10):1187-94.
(25) Goris RJ, te Boekhorst TP, Nuytinck JK, Gimbrere JS. Multiple-organ failure. Generalized autodestructive inflammation? Arch Surg 1985 Oct;120(10):1109-15.
(26) Rosman C, Westerveld GJ, Kooi K, Bleichrodt RP. Local treatment of generalised peritonitis in rats; effects on bacteria, endotoxin and mortality. Eur J Surg 1999 Nov;165(11):1072-9.
(27) Weisel CJ, Cederholm-Williams SA. Fibrinogen and fibrin. In: Domb AJ, Kost J, Wiseman DM, editors. Handbook of Biodegradable Polymers.Amsterdam: Harwood; 1997. p. 347-65.
(28) Morikawa T. Tissue sealing. American Journal Of Surgery 2001 Aug;182(2):29S-35S.
(29) Currie LJ, Sharpe JR, Martin R. The use of fibrin glue in skin grafts and tissue-engineered skin replacements: a review. Plastic And Reconstructive Surgery 2001 Nov;108(6):1713-26.
(30) Kumar A, Koenig KB, Johnson AR, Fair DS, Idell S. Inhibition of factor Xa-mediated procoagulant activity of human lung fibroblasts and pleural mesothelial cells. Eur Respir J 1995 Dec;8(12):2038-45.
(31) Schenone M, Furie BC, Furie B. The blood coagulation cascade. Curr Opin Hematol 2004 Jul;11(4):272-7.
(32) Hack CE. Tissue factor pathway of coagulation in sepsis. Crit Care Med 2000 Sep;28(9 Suppl):S25-S30.
(33) Bannerman DD, Goldblum SE. Mechanisms of bacterial lipopolysaccharide-induced endothelial apoptosis. Am J Physiol Lung Cell Mol Physiol 2003 Jun;284(6):L899-L914.
(34) Ajuebor MN, Flower RJ, Hannon R, Christie M, Bowers K, Verity A, et al. Endogenous monocyte chemoattractant protein-1 recruits monocytes in the zymosan peritonitis model. J Leukoc Biol 1998 Jan;63(1):108-16.
(35) Li FK, Davenport A, Robson RL, Loetscher P, Rothlein R, Williams JD, et al. Leukocyte migration across human peritoneal mesothelial cells is dependent on directed chemokine secretion and ICAM-1 expression. Kidney Int 1998 Dec;54(6):2170-83.
(36) Stout RD, Li Y, Miller AR, Lambe DW, Jr. Staphylococcal glycocalyx activates macrophage prostaglandin E2 and interleukin 1 production and modulates tumor necrosis factor alpha and nitric oxide production. Infect Immun 1994 Oct;62(10):4160-6.

(37) Zumla A. Superantigens, T cells, and microbes. Clin Infect Dis 1992 Aug;15(2):313-20.
(38) Ho-dac-Pannekeet MM. Peritoneal fluid markers of mesothelial cells and function. Adv Ren Replace Ther 1998 Jul;5(3):205-11.
(39) Bauer KA. Activation markers of coagulation. Best Practice & Research Clinical Haematology 1999 Sep;12(3):387-406.
(40) Coughlin SR. How the protease thrombin talks to cells. PNAS 1999 Sep 28;96(20):11023-7.
(41) Raftery AT. Regeneration of parietal and visceral peritoneum. A light microscopical study. Br J Surg 1973 Apr;60(4):293-9.
(42) Weisel JW. Fibrin assembly. Lateral aggregation and the role of the two pairs of fibrinopeptides. Biophysical Journal 1986 Dec;50(6):1079-93.
(43) Collen D, Lijnen HR. Basic and clinical aspects of fibrinolysis and thrombolysis. Blood 1991 Dec 15;78(12):3114-24.
(44) Risberg B, Eriksson E, Bjork S, Hansson GK. Immunohistochemical localization of plasminogen activators in human saphenous veins. Thromb Res 1986 Feb 1;41(3):301-8.
(45) Whawell SA, Vipond MN, Scott-Coombes DM, Thompson JN. Plasminogen activator inhibitor 2 reduces peritoneal fibrinolytic activity in inflammation. Br J Surg 1993 Jan;80(1):107-9.
(46) Wodzinski MA, Bardhan KD, Reilly JT, Cooper P, Preston FE. Reduced tissue type plasminogen activator activity of the gastroduodenal mucosa in peptic ulcer disease. Gut 1993 Oct;34(10):1310-4.
(47) Sitter T, Toet K, Fricke H, Schiffl H, Held E, Kooistra T. Modulation of procoagulant and fibrinolytic system components of mesothelial cells by inflammatory mediators. Am J Physiol 1996 Nov;271(5 Pt 2):R1256-R1263.
(48) van Hinsbergh VW, Kooistra T, Scheffer MA, Hajo vB, van Muijen GN. Characterization and fibrinolytic properties of human omental tissue mesothelial cells. Comparison with endothelial cells. Blood 1990 Apr 1;75(7):1490-7.
(49) Dunn DL, Simmons RL. Fibrin in peritonitis. III. The mechanism of bacterial trapping by polymerizing fibrin. Surgery 1982 Sep;92(3):513-9.
(50) Ahrenholz DH, Simmons RL. Fibrin in peritonitis. I. Beneficial and adverse effects of fibrin in experimental E. coli peritonitis. Surgery 1980 Jul;88(1):41-7.
(51) Hau T. Bacteria, toxins, and the peritoneum. World J Surg 1990 Mar;14(2):167-75.
(52) Ciano PS, Colvin RB, Dvorak AM, McDonagh J, Dvorak HF. Macrophage migration in fibrin gel matrices. Lab Invest 1986 Jan;54(1):62-70.
(53) Hau T, Nishikawa RA, Phuangsab A. The effect of bacterial trapping by fibrin on the efficacy of systemic antibiotics in experimental peritonitis. Surg Gynecol Obstet 1983 Sep;157(3):252-6.
(54) Rotstein OD, Pruett TL, Simmons RL. Fibrin in peritonitis. V. Fibrin inhibits phagocytic killing of Escherichia coli by human polymorphonuclear leukocytes. Ann Surg 1986 Apr;203(4):413-9.



Chapter 2

A peritonitis model with low mortality and persisting intra-abdominal abscesses

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ABSTRACT

Background
Intra-abdominal abscesses are a potential source of recurrent or residual infection after surgical intervention for secondary peritonitis. The development of therapies requires a model which combines low mortality with the formation of persisting abscesses and which is also suitable to study the local inflammatory response.

Methods:
Wistar rats were injected intraperitoneally with sterile rat feces, increasing doses of E. coli (10^4-10^8 cfu/ml) and a fixed dose of B. Fragilis (10^4 cfu/ml). After one h a laparotomy was performed and the peritoneal cavity was debrided. Blood samples were taken after 6 and 24 h. Abdominal fluid samples were collected after 24 and 72 h. The rats were killed after 5 days and the abdomen was inspected for abscesses.

Results:
Mortality was 90% in the two groups with the highest doses of E. coli and 30% in those with the two lowest doses. In the latter groups all surviving rats but one showed intra-abdominal abscesses and bacteremia was encountered frequently, especially after 24 h in the 10^5 cfu E. coli group. The groups receiving 10^4 - 10^6 cfu E.coli showed similar plasma IL-6 concentrations after 6 h which lowered significantly after 24 h. No circulating TNF-α was found. Considerable concentrations of TNF-α, IL-6, IL-1β and IL-10 were found in the peritoneal fluid after 24 h. At 72 h cytokine levels were reduced significantly and remained the highest in the animals dosed with 10^6 cfu E. coli.

Conclusions:
The present model is suitable to study the mechanisms involved in, and prevention of, intra-abdominal abscess formation after surgical treatment of generalized peritonitis.

INTRODUCTION

Generalized peritonitis is a life threatening disease caused, in the majority of cases, by perforation of the digestive tract. Sometimes, this disease can be controlled by local defense mechanisms: formation of fibrinous adhesions, clearance of bacteria via lymphatic stomata in the diaphragm and the cellular defenses of the peritoneal cavity(1;2). However, if these mechanisms fail, the peritoneal cavity is flooded with bacteria, inflammatory mediators and visceral contents. It will turn into one large inflamed compartment in which the local defense mechanisms may become detrimental to the patient.
Fibrin deposition on the diaphragm may prevent the clearance of bacteria from the abdominal cavity by occluding the lymphatic stomata, thereby containing the infection. However, entrapment in fibrin clots renders bacteria unreachable for phagocytes and antibiotic therapy(3-5). Shortage of oxygen in the peritoneal fluid and neutrophil activation by complement, bacteria trapped in fibrin and visceral contents, will cause extracellular release of lysozymal enzymes. These are capable of digesting normal and viable tissue resulting not only in necrosis but also in continued stimulation of inflammation and influx of neutrophils(6). This entire process may readily become self-perpetuating, even in the absence of bacteria(2;7;8). The outcome will be fatal without surgical intervention(9).
Removal of the source of contamination, debridement of the abdominal cavity, proper antibiotics and drainage of abscesses are the cornerstones of successful surgical therapy in generalized peritonitis. However, in a considerable number of cases adequate therapy still results in the formation of residual abscesses, which carry a substantial mortality and morbidity (2). Recently, a mortality rate of 30% was reported in ICU patients with ongoing intra-abdominal infection; it can be as high as 50% if recurrent infection occurs(10).
The search for further treatment options is best performed in an experimental model, which reproducibly combines a low mortality with the formation of persisting, preferably multiple, abscesses with time. In addition, multiple measurements of local inflammatory mediators would contribute to our understanding of the pathophysiology. An intraperitoneal bolus challenge with Escherichia coli (E. coli) and Bacteroides fragilis (B. fragilis) is believed to initiate events which are a valid representation of the course of secondary peritonitis(11). To our knowledge, models described in the literature have not yet incorporated all of the requirements mentioned above. The aim of the present study was to develop such a model, which allows for the the study of mechanism involved in, and prevention of, intra-abdominal abscess formation after surgical treatment of generalized peritonitis.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 250-280 g, (Harlan BV, Horst, the Netherlands) were housed two per cage and accustomed to laboratory conditions for 1 week before the start of the experiment. All animals were weighed daily and had free access to water and standard rodent chow throughout the entire experimental period (Hope Farms, Woerden, the Netherlands). The Animal Ethics Review Committee of the Radboud University Nijmegen, approved the study.

Fecal suspension

Fresh feces from Wistar rats were collected and suspended in twice the volume of sterile, pyrogen-free water. The suspension was sterilized in an autoclave. After centrifugation (15 min at 750 x g), the upper two layers were combined, autoclaved again and stored at 4°C. At the day of the experiment 1 ml of fecal suspension was mixed with 0.5 ml of Schaedler solution containing either 10^4, 10^5, 10^6, 10^7 or 10^8 cfu E. coli, strain 25922 from the American Type Culture Collection. Immediately before inoculation 0.5 ml of Schaedler solution containing 10^4 cfu from a clinical B. fragilis isolate was added to the fecal inoculum as well. Bacterial concentrations were determined by densitometry and nephelometry (BD; Crystalspec, Franklin Lakes, NJ, USA) at the day of inoculation.

Experimental design

Sixty animals were randomly divided into six groups of ten rats. Peritonitis was induced in five groups of rats by percutaneous intra-abdominal injection in the right lower quadrant of 2 ml of a fecal suspension containing 10^4 cfu B. fragilis and 10^4, 10^5, 10^6, 10^7 or 10^8 cfu E. coli, respectively. The control group was injected with sterilized faeces only.

One h after inoculation, the rats were anesthetized by inhalation of a mixture of isoflurane-oxygen-nitrous oxide. The abdominal cavity was debrided and rinsed with 5 ml of sterile saline of 38°C. During debridement (partial) omentectomy was performed in order to remove macroscopic omental contamination. The abdomen was closed with a running suture of 4.0 Polyglactin 910 (Vicryl®, Ethicon, Amersfoort, The Netherlands), the skin was closed with staples. Rats received 5 ml normal saline subcutaneously for resuscitation.

Blood samples were taken at 6 and 24 h after inoculation by cardiac puncture. Peritoneal fluid

samples were taken after 24 and 72 h via a laparotomy. After 5 days the animals were killed by CO_2 asphyxiation. The abdomen was inspected, by the same persons who were unaware of the treatment, with particular attention to the number and location of abscesses.

Sample collection

After inducing anaesthesia with an isoflurane-oxygen-nitrous oxide mixture, 1 ml blood samples were taken by cardiac puncture and collected in tubes containing 50 IU heparin. Part was used for bacterial culturing and the remainder was centrifuged for 10 min at 1600 x g. The plasma was stored at -80°C for cytokine assays (see below). Peritoneal fluid samples were also collected under anaesthesia: After reopening the abdomen, 5 ml of sterile saline of 38°C was installed in the abdominal cavity. The abdomen was gently massaged and at least 2 ml of fluid were withdrawn. The samples were centrifuged for 10 min at 750 x g and supernatants stored at -80°C until further analysis.

Sample analysis

Heparinized blood samples (300 µl) were cultured for the presence of E. coli. Serial dilutions in Brain Heart Infusion broth were plated onto MacConkey blood agar plates. After 24 and 48 h of incubation at 37°C bacteria were counted and identified.

Interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were measured in plasma as well as in peritoneal fluid. In addition to these cytokines, interleukin-1β (IL-1β) and interleukin-10 (IL-10) were measured in peritoneal fluid. All assays were performed using commercially available ELISA kits (Endogen®, Pierce Biotechnology Inc, Rockford IL, USA). According to the manufacturer, the sensitivities for the various assays were < 10 pg/ml for TNF-α, < 12 pg/ml for IL-1β, < 16 pg/ml for IL-6 and < 3 pg/ml for IL-10, respectively. Cells were counted in peritoneal fluid, after dilution of the sediment with saline (to a concentration of 50.000 cells/ml) and staining with May-Grunwald Giemsa reagent.

Statistics

Statistical analysis was performed using Tukey Kramer multiple group comparisons. Kruskal-Wallis and Mann-Whitney test were applied to process nonparametric data. Survival rates were compared by Kaplan Meier Survival analysis. P values of < 0.05 are considered significant.

RESULTS

Survival

All rats in the control group survived the experimental period. In the rats that received E. coli and B. fragilis, survival was significantly lower than in the control group and steadily decreased with the bacterial load (Figure 1). Survival was 70% in groups receiving 10^4 or 10^5 cfu E. coli, 40% in the group with 10^6 cfu E. coli and 10% after receiving either 10^7 or 10^8 cfu E. coli, respectively.

Clinical course

All rats became severely ill after inoculation, as was evident from lack of movement, erect body hair and anorexia. Body weight decreased significantly within 24 h after inoculation and stabilized thereafter. Weight course was similar in the control group and the groups which had received 10^4 - 10^6 cfu E. coli (Figure 2).

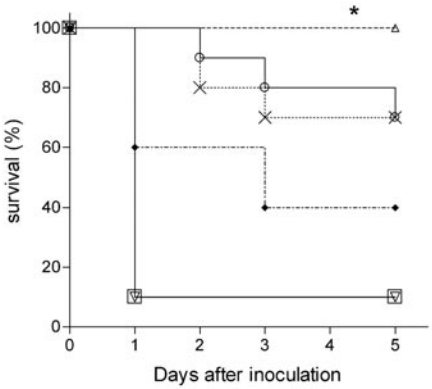

Figure 1. **Survival.**
Survival in six groups of rats inoculated on day 0 with sterile faeces (controls) or sterile faeces mixed with a fixed dose of B. fragilis and increasing doses of E. coli. △: controls; ○: 10^4 cfu E. coli; ×: 10^5 cfu E. coli; ◆: 10^6 cfu E. coli; □ 10^7 cfu E. coli; ▽: 10^8 cfu E. coli.

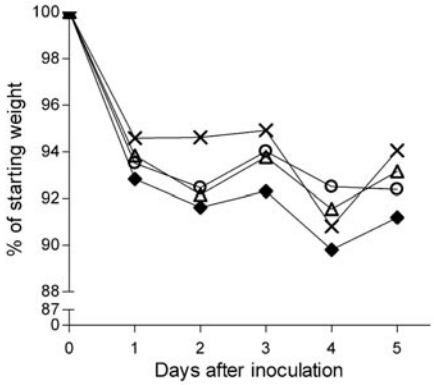

Figure 2. **Course of body weight.**
Daily body weight in four groups of rats inoculated on day 0 with sterile faeces (controls) or sterile faeces mixed with a fixed dose of B. fragilis and increasing doses of E. coli. Average weight is expressed as percentage of the weight at inoculation. △: controls; ○: 10^4 cfu E. coli; ×: 10^5 cfu E. coli; ◆: 10^6 cfu E. coli. The results of the groups receiving 10^7 or 10^8 cfu E. coli are not shown since only 1 rat survived in each group.

Abscess formation

None of the rats in the control group, inoculated with sterile faeces only, developed abscesses. Fifteen out of 18 survivors in the peritonitis groups inoculated with 10^4 - 10^6 cfu E. coli showed intra-abdominal abscesses after 5 days (Table 1). The median number of abscesses per animal did not differ significantly between these groups: 2, 2.5 and 1.5 after 10^4, 10^5 or 10^6 cfu E. coli, respectively.

Blood cultures

Blood cultures from the control group were never positive for E. coli. Six h after inoculation, when all rats were still alive, cultures were already positive in a majority of rats in the groups which had received 10^6 cfu E. coli or more (Table 2). Median concentrations were 0 cfu/ml in the groups receiving 10^4 or 10^5 cfu E. coli and 50, 2050 and 5300 cfu/ml after inoculation with 10^6, 10^7 or 10^8 cfu E. coli, respectively. After 24 h, frequency of positive blood cultures increased. Median concentrations increased from 0 to 1200 cfu/ml in the 10^5 cfu E. coli group and from 50 to 1700 cfu/ml in the group which had received 10^6 cfu E. coli.

	Survivors with abcesses	Number of abcesses/rat
Controls	0/10 (0%)	
104 cfu E. coli	7/7 (100%)*	1 - 3
105 cfu E. coli	6/7 (86%)*	1 - 4
106 cfu E. coli	2/4 (50%)	1 - 2

Table 1. **Abscess formation.**
Abscesses were counted five days after inoculation. The controls were inoculated with sterile faeces only, the experimental groups with sterile faeces, 10^4 cfu B. fragilis and increasing doses of E. coli. * $p<0.05$ vs controls.

	6 h	24 h
Controls	0/10	0/10
10^4 cfu E. coli	0/10	2/10
10^5 cfu E. coli	1/10	9/10
10^6 cfu E. coli	7/10	6/6
10^7 cfu E. coli	8/10	0/1
10^8 cfu E. coli	9/10	2/2

Table 2. **Frequency of bacteremia.**
The controls were inoculated with sterile faeces only, the experimental groups with sterile faeces, 10^4 cfu B. fragilis and increasing doses of E. coli.

Peritoneal cells and cytokines

Differential cell counts were performed in the abdominal fluid collected from the control group and the group which had received 10^6 cfu E. coli. The percentage of neutrophils in the control group went down from 74 ± 9 (mean ± SD, n=4) at 24 h to 20 ± 12 at 120 h. The same happened in the experimental group: 66 ± 13 to 16 ± 9 %. Over the same period the percentage of eosinophils rose from 5 ± 2 to 55 ± 22 % in the controls and from 9 ± 9 to 39 ± 5 % in the group which had received additional E. coli. Monocytes were 21 ± 8 % and 23 ± 10 % in the controls and 25 ± 7 vs 43 ± 8 % in the experimental groups. Absolute numbers of cells did not differ between groups.

Cytokine concentrations were measured in peritoneal fluid, collected after 24 or 72 h, from rats from the control group and the groups which had received 10^4 -10^6 cfu E. coli.

At 24 h substantial levels of TNF-α were found in all groups and after 72 h concentrations were reduced. There were no significant differences between groups (Figure 3A). If all values were taken together, the median value (range) was 115 (0-359) pg/ml after 24 h and 0 (0-295) pg/ml after 72 h. For IL-6, equivalent concentrations were also measured in the four groups at 24 h after inoculation.

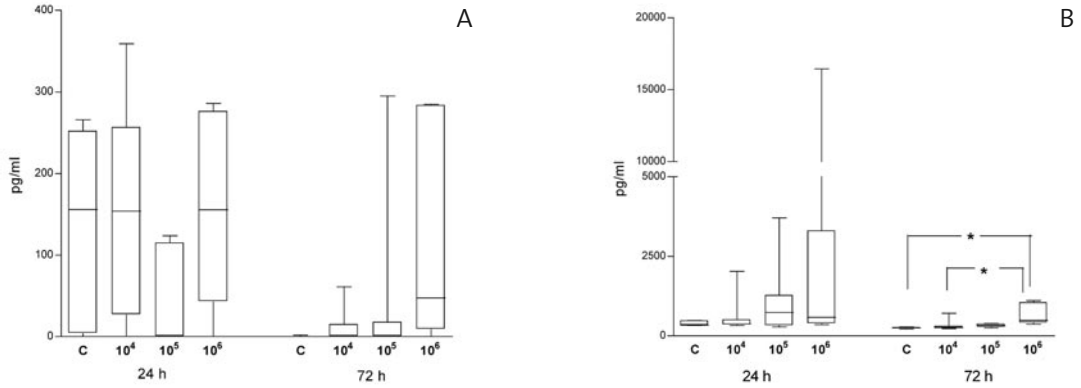

Figure 3a en 3b. **TNF-α and IL-6 in peritoneal fluid.**
TNF-α (A) and IL-6 (B) concentrations in peritoneal fluid collected at 24 and 72 h after inoculation in the control group and the groups receiving 10^4, 10^5 or 10^6 cfu E. coli. Data are depicted as medians with 25-75 percentiles in boxes and 5-95 percentiles represented as vertical lines. *: $p < 0.05$.

The rather wide range of the values observed in the groups which had received live bacteria was caused in each case by values found in a single animal. The overall median concentration was 464 (range: 282-16444) pg/ml. After 72 h concentrations were significantly lower. Those in the group which had received 10^6 cfu E. coli remained significantly ($p<0.05$) higher than those in the control group and the group with 10^4 cfu E. coli (Figure 3B). A rather similar picture was obtained for IL-1β and IL-10 (Figure 4). There were no significant differences between groups after 24 h: taken together the median concentrations were 312 (30-6000) pg/ml for IL-1β and 254 (26-1400) pg/ml for IL-10. In all groups, values decreased significantly between 24 and 72 h. At the latter time point, the group which had received the highest dose of E. coli again showed the highest concentrations, though not always significantly so.

Plasma cytokines

Plasma samples, collected at 6 and 24 h, were analyzed for both TNF-α and IL-6. TNF-α levels were below the detection limit (<10 pg/ml) in all samples. At 6 h, IL-6 was present in plasma of all but one animal, independent of the group (Figure 5). The overall median concentration was 495 (0-1620) pg/ml. At this time point, median plasma concentrations in 4 rats from the groups which had received 10^7 or 10^8 cfu E. coli were 1873 and 2291 pg/ml, respectively. After 24 h, plasma IL-6 was significantly lower than at 6 h in all groups and tended to be higher in the groups with the higher doses of E. coli.

DISCUSSION

Inoculation of a mixture of sterilized feces, B. fragilis and E.coli in the abdominal cavity of rats followed by surgical debridement after 1 h, provides a reproducible model to study residual abscesses after treatment of generalized peritonitis.

To study the pathophysiology and treatment modalities of intra-abdominal abscesses, an experimental model with low mortality and a high rate of abscesses is needed. Mortality in the present model was closely correlated to the amount of E. coli given. At the lower doses of E. coli (10^4-10^5 cfu) the survival was 70-80% accompanied by 86-100% intra-abdominal abcesses. With the exception of the group receiving the lowest dose of E. coli, bacteremia occured almost invariably within the first 24 h after the onset of peritonitis.

The current model also includes the sampling of peritoneal fluid at 24 and 72 h after bacterial inoculation, in order to measure local inflammatory mediators. For this purpose additional laparotomies

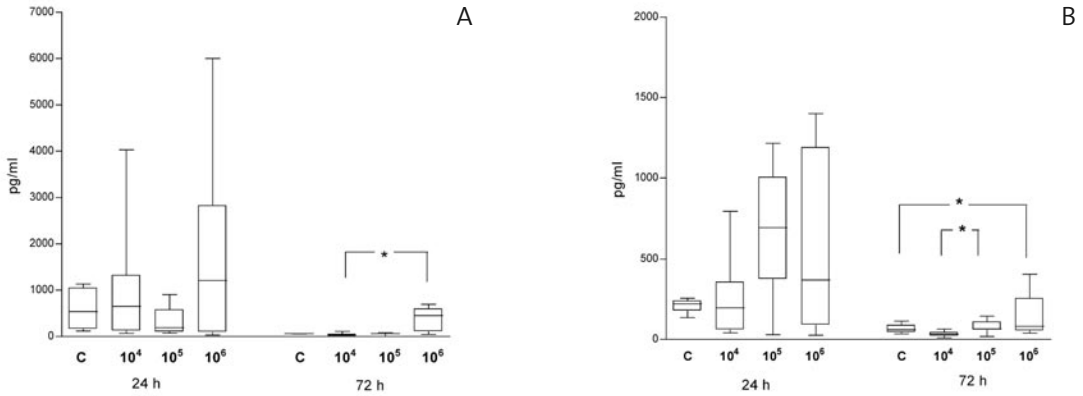

Figure 4a en 4b. **IL-1β and IL-10 in peritoneal fluid.**
IL-1β (A) and IL-10 (B) concentrations in peritoneal fluid collected at 24 and 72 h after inoculation in the control group and the groups receiving 10^4, 10^5 or 10^6 cfu E. coli. Data are depicted as medians with 25-75 percentiles in boxes and 5-95 percentiles represented as vertical lines. *: $p < 0.05$.

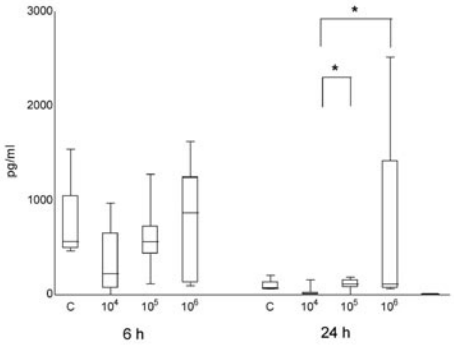

Figure 5. **IL-IL-6 in plasma.**
IL-6 concentrations in plasma collected at 6 and 24 h after inoculation in the control group and the groups receiving 10^4, 10^5 or 10^6 cfu E. coli. Data are depicted as medians with 25-75 percentiles in boxes and 5-95 percentiles represented as vertical lines. *: $p < 0.01$

were performed since this allowed the most reproducible method for extraction of fixed volumes of fluid from the abdominal cavity. Animals recovered quite quickly and without any complications from the short periods of anesthesia necessary (also at 6 h in order to sample blood). The course of body weight indicates that they only lost weight in the first 24 h after inoculation as a result of severe and immediate illness due to the primary bacterial insult. Still, it should be emphasized that the outcome of the model is the combined result of all procedures described and that no attempt was made, since this would have exceeded the purpose of the present study, to investigate the contribution of the individual parts. A small pilot study preceding the experiment described here showed that inoculation with saline only, and perfomance of all subsequent procedures, did not result in any pathology at all (data not shown).

So far, the animal models used to study intra-abdominal abscesses are based on either peritoneal contamination with endogenous bacteria using the cecal ligation and puncture (CLP) model or intraperitoneal application of exogenous bacteria, mostly in combination with adjuvant substances. Both experimental models are relevant to the formation of residual abscesses in rats with generalized peritonitis.

The CLP model is a well-defined experimental model to induce peritonitis. In rats, the cecum is ligated and punctured with a needle, causing ischemia of the cecum and leakage of its contents into the abdominal cavity. The severity of the peritonitis is determined by the diameter of the needle used. If CLP is followed by cecal resection 12 or 24 h afterwards, mortality can be limited and persisting abscesses will be found. Mortality rates between 0 and 28 % with - mostly single - residual abscesses in 83% of rats have been reported(12). The model mimics the clinical situation of postoperative peritonitis very well causing a generalized peritonitis by endogenous flora of the rat. However, cecal ischemia plays a significant role and will influence the inflammatory response. Therefore, the CLP model appears to be more appropriate to study generalized inflammatory responses and sepsis than the local inflammatory responses(13-15). Moreover, in this CLP model surgical therapy is performed quite some time after the onset of bacterial contamination of the peritoneum, which is often not the case, and never the intention, in clinical practice. The above is not meant to suggest that the current model is without a systemic response: both the -transient - presence of bacteria and IL-6 in the circulation prove otherwise.

Instillation of a standardized mixture of bacteria most frequently isolated in secondary peritonitis together with fecal material mimics the situation of a secondary generalized peritonitis after

perforation of the gut(9). In contrast to CLP, peritonitis is caused by bacteria from clinical isolates, that do not belong to the endogenous flora of the rats, which may influence the immune response. While several authors have reported on abscess formation after intra-abdominal bacterial inoculation, none of the studies mentioned below have included all requirements for such a model (e.g. multiple abscesses and measurement of local mediators), as stated in the introduction. Rosman et al. inserted a rat faecal suspension mixed with 10^4 cfu E. coli and 10^4 cfu B. fragilis into the peritoneal cavity of rats, resulting in abscesses in all animals and a mortality of 30%(16). These numbers are in agreement with our observations. Rotstein and Kao implanted a human fibrin clot containing 2×10^7 cfu E. coli and 2×10^8 cfu B. fragilis into the abdominal cavity of rats via a laparotomy. All rats developed a single abscess accompanied by a mortality of 41%(17). Wandall et al. implanted a gelatin capsule filled with a mixture of 2.2×10^6 cfu or less E. coli, B. fragilis, sterile feces and barium sulphate and found a single abscess in all rats without mortality(18). In our study the mortality in the group with 10^6 cfu E. coli was 60%. Possibly, the gelatin capsule may provide a slower release of bacteria leading to a more protracted and less severe course of disease. Their study showed the response to intra-abdominal bacterial challenge to be reproducible and dose-dependent with respect to mortality, weight change, food intake and bacteremia. No effort was made to measure the local inflammatory response. The rationale for adding barium sulphate, which obviously is absent during secondary peritonitis in humans and is known to act as an adjuvant to the inflammatory response, remains unexplained(18). The local inflammatory response was quantified by measuring levels of the pro-inflammatory cytokines TNF-α, IL-6 and IL-1β and the anti-inflammatory IL-10. The former cytokines are known to induce acute inflammation of the peritoneum(19;20) and are correlated with adverse outcome(21-23), whereas the latter is known to suppress the production of pro-inflammatory cytokines(19). At 24 h after induction of peritonitis, when a strong local inflammatory reaction may be expected, significant concentrations of all cytokines are indeed present in the abdominal fluid. At this time point the systemic reaction is much more subdued as reflected by the far lower plasma concentrations for IL-6 while TNF-α was even absent from the circulation. It may be that these cytokines are cleared much quicker from the blood stream than from the peritoneal cavity. Also, the peritoneal cavity may be a source of protracted cytokine production.

Several other groups have studied the local and systemic inflammatory effects of bacterial peritonitis. Sewnath induced peritonitis by a single intraperitoneal injection of a E. coli /saline suspension(24). Montravers used a gelatin capsule filled with a mixture of E. coli and B. fragilis and added barium

sulphate(25). The group of Martineau employed a 12 h continuous intra-abdominal infusion with a E. coli/fecal suspension(26). Although these studies differ considerably from each other and ours regarding the way of inducing peritonitis and the dose of bacteria given, the results are rather consistent. Strongly elevated TNF-α and IL-6 levels in the abdominal fluid within 24 h after instillation of the bacteria are found together with a much lesser increase in plasma levels of both cytokines. In our model both local and systemic IL-6 and TNF-α levels were lower than those reported in the studies mentioned above. This may in part be explained by dilution, since we took the peritoneal samples after instillation of 5 ml normal saline in the abdomen.

The elevated cytokine levels in the rats that received fecal suspension alone probably are the result of the endotoxin present. A semi-quantitative analysis of the suspension confirmed the presence of significant concentrations of lipopolysaccharide (data not shown). It may seem somewhat surprising that, at least after 24 h, peritoneal cytokine levels were similar in the control group and the groups receiving increasing doses of E. coli. Interestingly, at 72 h, when the cytokine levels are reduced and clinical signs of illness strongly decreased, there seems to be a certain degree of correlation between bacterial load and abdominal cytokine concentration. It may be that, during the first phase of illness, locally produced LPS binding protein, which is an essential component in the innate immune response to E. coli peritonitis(27;28), is able to reduce the free endotoxin to similar levels in the different groups. As a consequence, mortality does not correlate with the local concentration of inflammatory mediators but seems to be linked to the bacterial counts in the circulation, as measured after both 6 and 24 h.

The present model has provided satisfactory results regarding abscess formation, survival and local cytokine response in an animal model for secondary peritonitis. This model is suitable to study the mechanisms involved in intra-abdominal abscess formation after surgical treatment of generalized peritonitis. Further experiments will be directed at fibrinolytic therapy as a means to reduce, and possibly prevent, the formation of persisting abscesses.

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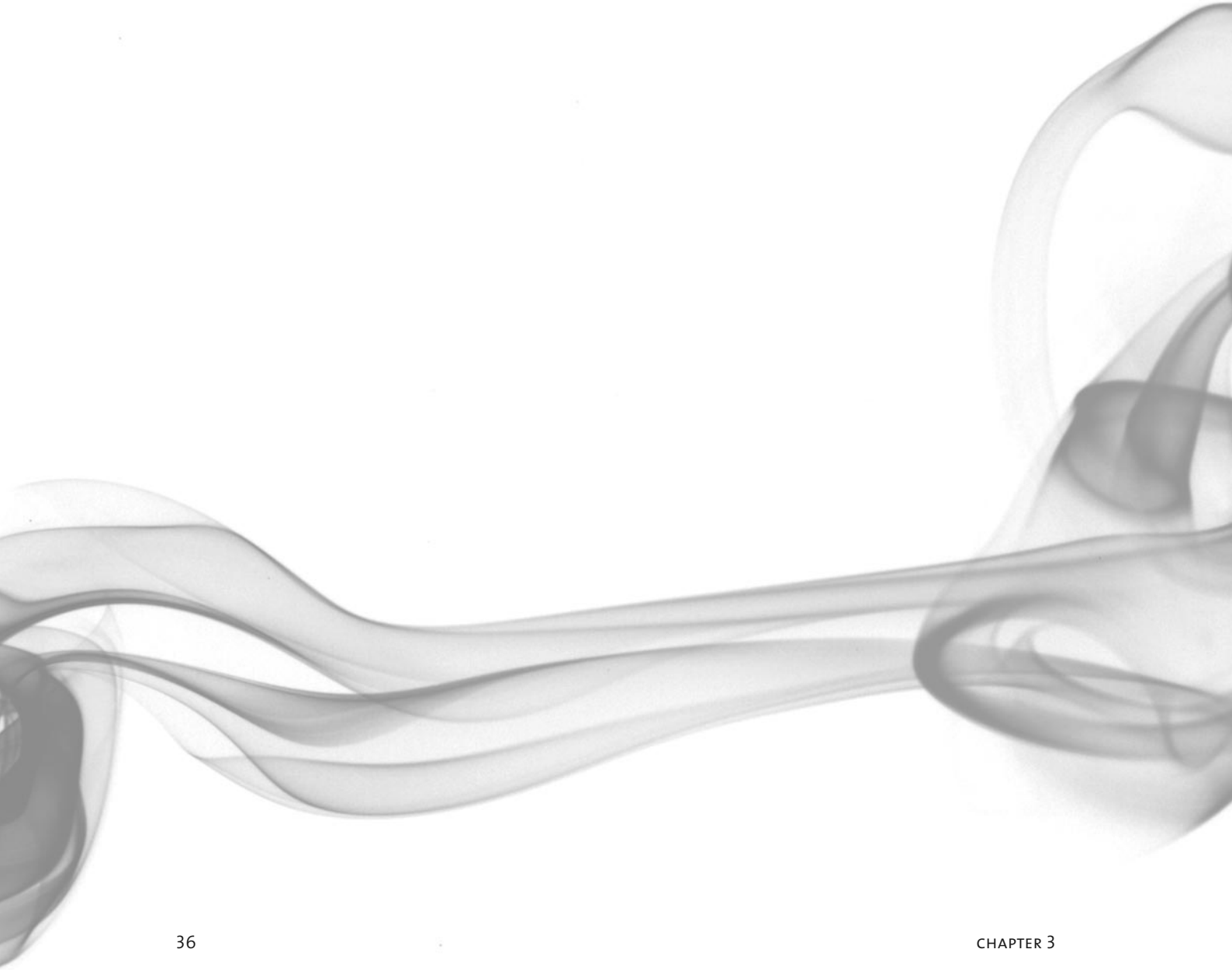
REFERENCE LIST

(1) Farthmann EH, Schoffel U. Principles and limitations of operative management of intraabdominal infections. World J Surg 1990 Mar;14(2):210-7.
(2) Farthmann EH, Schoffel U. Epidemiology and pathophysiology of intraabdominal infections (IAI). Infection 1998 Sep;26(5):329-34.
(3) Rotstein OD. Role of fibrin deposition in the pathogenesis of intraabdominal infection. Eur J Clin Microbiol Infect Dis 1992 Nov;11(11):1064-8.
(4) Heemken R, Gandawidjaja L, Hau T. Peritonitis: pathophysiology and local defense mechanisms. Hepatogastroenterology 1997 Jul;44(16):927-36.
(5) Hall JC, Heel KA, Papadimitriou JM, Platell C. The pathobiology of peritonitis. Gastroenterology 1998 Jan;114(1):185-96.
(6) Hau T. Bacteria, toxins, and the peritoneum. World J Surg 1990 Mar;14(2):167-75.
(7) Holzheimer RG, Schein M, Wittmann DH. Inflammatory response in peritoneal exudate and plasma of patients undergoing planned relaparotomy for severe secondary peritonitis. Arch Surg 1995 Dec;130(12):1314-9.
(8) Laroche M, Harding G. Primary and secondary peritonitis: an update. Eur J Clin Microbiol Infect Dis 1998 Aug;17(8):542-50.
(9) Nathens AB. Relevance and utility of peritoneal cultures in patients with peritonitis. Surg Infect (Larchmt) 2001;2(2):153-60.
(10) Marshall JC, Innes M. Intensive care unit management of intra-abdominal infection. Crit Care Med 2003 Aug;31(8):2228-37.
(11) Parker SJ, Watkins PE. Experimental models of gram-negative sepsis. Br J Surg 2001 Jan;88(1):22-30.
(12) Reijnen MMPJ, Skrabut EM, Postma VA, Burns JW, van Goor H. Polyanionic Polysaccharides Reduce Intra-abdominal Adhesion and Abscess Formation in a Rat Peritonitis Model. Journal of Surgical Research 2001 Dec;101(2):248-53.
(13) Ferri LE, Pascual J, Seely AJ, Giannias B, Christou NV. Intra-abdominal sepsis attenuates local inflammation-mediated increases in microvascular permeability at remote sites in mice in vivo. Surgery 2004 Feb;135(2):187-95.
(14) Singleton KD, Wischmeyer PE. Distance of cecum ligated influences mortality, tumor necrosis factor-alpha and interleukin-6 expression following cecal ligation and puncture in the rat. Eur Surg Res 2003 Nov;35(6):486-91.
(15) Maier S, Traeger T, Entleutner M, Westerholt A, Kleist B, Huser N, et al. Cecal ligation and puncture versus colon ascendens stent peritonitis: two distinct animal models for polymicrobial sepsis. Shock 2004 Jun;21(6):505-11.
(16) Rosman C, Westerveld GJ, Kooi K, Bleichrodt RP. Local treatment of generalised peritonitis in rats; effects on bacteria, endotoxin and mortality. Eur J Surg 1999 Nov;165(11):1072-9.
(17) Rotstein OD, Kao J. Prevention of intra-abdominal abscesses by fibrinolysis using recombinant tissue plasminogen activator. J Infect Dis 1988 Oct;158(4):766-72.
(18) Wandall DA, Arpi M, Wandall JH. A rat model of non-lethal bacterial infection. APMIS 1997 Mar;105(3):187-91.
(19) Haupt W, Riese J, Denzel C, Zowe M, Gusinde J, Siassi M, et al. Culture of human peritoneum - a new method to measure the local cytokine response and the effect of immunomodulators. Infection 1998 Sep;26(5):345-8.

(20) Margetts PJ, Kolb M, Yu L, Hoff CM, Holmes CJ, Anthony DC, et al. Inflammatory Cytokines, Angiogenesis, and Fibrosis in the Rat Peritoneum. Am J Pathol 2002 Jun 1;160(6):2285-94.
(21) Schein M, Wittmann DH, Holzheimer R, Condon RE. Hypothesis: compartmentalization of cytokines in intra-abdominal infection. Surgery 1996 Jun;119(6):694-700.
(22) Oymak Y, Velioglu A, Haklar G, Ozener C, Yalcin AS. Early detection of peritonitis in continuous ambulatory peritoneal dialysis patients by cytokine measurements. Clin Chem Lab Med 2003 May;41(5):640-5.
(23) Hirabayashi N, Tanimura H, Yamaue H. Nitrite/nitrate oxide and cytokines changes in patients with surgical stress. Dig Dis Sci 2005 May;50(5):893-7.
(24) Sewnath ME, Olszyna DP, Birjmohun R, ten Kate FJ, Gouma DJ, van der Poll T. IL-10-deficient mice demonstrate multiple organ failure and increased mortality during Escherichia coli peritonitis despite an accelerated bacterial clearance. J Immunol 2001 May 15;166(10):6323-31.
(25) Montravers P, Maulin L, Mohler J, Carbon C. Microbiological and inflammatory effects of murine recombinant interleukin-10 in two models of polymicrobial peritonitis in rats. Infect Immun 1999 Apr;67(4):1579-84.
(26) Martineau L, Shek PN. Peritoneal cytokine concentrations and survival outcome in an experimental bacterial infusion model of peritonitis. Crit Care Med 2000 Mar;28(3):788-94.
(27) Knapp S, de Vos AF, Florquin S, Golenbock DT, van Der PT. Lipopolysaccharide binding protein is an essential component of the innate immune response to Escherichia coli peritonitis in mice. Infect Immun 2003 Dec;71(12):6747-53.
(28) Schafer K, Schumann RR, Stoteknuel S, Schollmeyer P, Dobos GJ. Lipopolysaccharide-binding protein is present in effluents of patients with Gram-negative and Gram-positive CAPD peritonitis. Nephrol Dial Transplant 1998 Apr;13(4):969-74.



Chapter 3

Tissue-type plasminogen activator prevents formation of intra-abdominal abscesses after surgical treatment of secondary peritonitis in a rat model

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ABSTRACT

Background
Optimal therapy of secondary peritonitis frequently results in the formation of residual abscesses, which bear a substantial mortality and morbidity. This study aims to prove that fibrinolytic therapy with recombinant tissue plasminogen activator (rtPA) can reduce abscess formation after surgical treatment of secondary peritonitis in a rat model, without causing unwanted side-effects.

Methods:
Male Wistar rats received an intra-abdominal injection with a suspension of sterile feces, 10^5 cfu E. coli and 10^4 cfu B. fragilis. Surgical debridement was performed 1 h after inoculation. Animals were randomized into 4 groups (n=14 each). Three groups received human rtPA at 1 h (rtPA1), 1 h and 6 h (rtPA2) and 1, 6 and 24 h (rtPA3), respectively. Each dose contained 1.25 mg rtPA. Controls received saline only. Animals were killed after 5 days.

Results:
rtPA treatment reduced abscess formation in surviving animals, depending on number of doses given. Animals in group rtPA3 had no abscesses in contrast to 88% of the controls (mean 3.6 ± 2.7 abscesses per rat; $p<0.05$). In the rtPA1 and rtPA2 group frequency of abscess formation was 58% and 33%, respectively. Mortality, course of body weight and bacteremia were not affected by rtPA and neither were peritoneal cell counts and levels of TNF-α, IL-1β, IL-6 and IL-10. No bleeding complications were observed.

Conclusions:
rtPA reduces intra-abdominal abscess formation after surgical treatment of generalized peritonitis without increasing mortality or affecting the local inflammatory response.

INTRODUCTION

Secondary peritonitis, which is by far the most common form of peritonitis, results from the loss of integrity of the gastrointestinal or genitourinary tract, leading to contamination of the peritoneal space(1). Despite improved diagnostic modalities, potent antibiotics, modern intensive care, and aggresive surgical treatment, up to one third of patients still die from generalized peritonitis(2;3). Removal of the source of contamination, debridement of the abdominal cavity and drainage of abscesses are the cornerstones of successful surgical therapy in generalized peritonitis. Antibiotics reduce the general effects of sepsis and infectious complications(4;5). However, optimal therapy including antibiotic treatment results in the formation of residual abscesses in a considerable number of cases, which bear a substantial mortality and morbidity(3;6).

In peritonitis both the coagulation and fibrinolytic cascade are upregulated. In the early phase of peritonitis, coagulation dominates, resulting in the formation of fibrin in the abdominal cavity. These fibrin deposits contain viable bacteria that become unreachable for the local immune defenses in the abdominal cavity and for antibiotics(7;8). Hence, these contaminated fibrin clots form the nidus for intra-abdominal abcesses(8). In addition, fibrin deposits may become infiltrated by fibrocytes and become fibrous adhesions which by itself may result in considerable morbidity and even mortality(9). Thus, well-timed therapy aiming at restoration of intraperitoneal fibrinolytic capacity may prevent the formation of residual abscesses and adhesions.

Tissue-type plasminogen activator (tPA), the main physiological plasminogen activator, has been applied as a fibrin-specific thrombolytic agent for the treatment of thromboembolic diseases(10). For instance, tPA has been succesfully applied for the treatment of ischemic stroke(11) pulmonary embolism(12) and catheter thrombosis(13). Its recombinant form, rtPA, has been proven effective in the treatment of pleural empyema(14) and in two clinical cases of peritonitis(15;16).

The rationale behind the use of rtPA in peritonitis is early breakdown of fibrin clots to eliminate the nidus for residual abscesses, without the risk of hemorrhage due to systemic activation of plasminogen activator inhibitor as a result of sepsis. In previous experimental studies, rtPA was effective in reducing abscess formation in rodent models of peritonitis without any apparent increase in bleeding complications(17;18). However, reduced abscess formation was associated with a considerable and significantly increased mortality, which may be due to release of bacteria from the abdominal cavity, leading to bacteriaemia, sepsis and/or an exaggarated local immune response(17;19). The

present study was initiated to determine whether the effect of intra-abdominal application of rtPA can be optimized by changing the method of application and the dosage scheme. In addition, it was studied whether intraperitoneal rtPA therapy is associated with an increased inflammatory response in the abdominal cavity, increased incidence and severity of bacteriaemia or mortality.

MATERIALS AND METHODS

Experimental design

Fifty-five animals were randomly divided into three groups of 14 and one group of 13 rats. Peritonitis was induced in all rats by intraperitoneal injection of 2 ml of a fecal suspension containing 10^4 cfu B. fragilis (from a clinical isolate) and 10^5 cfu E. coli (strain 25922 from the American Type Culture Collection), respectively(20).

One hour after inoculation, the rats were anesthetized by inhalation of a mixture of isoflurane-oxygen-nitrous oxide. The abdominal cavity was debrided including a partial resection of grossly contaminated omentum and rinsed with 5 ml of sterile saline of 38°C. The abdomen was closed with a running suture of 4.0 Polyglactin 910 (Ethicon, The Netherlands), the skin was closed with staples. Rats received 5 ml normal saline subcutaneously for resuscitation.

Three experimental groups received intraperitoneal injections with recombinant human tPA at 1 h (rtPA1), 1 h and 6 h (rtPA2) and 1, 6 and 24 h (rtPA3) after inoculation, respectively. Each dose consisted of 1.25 mg rtPA (Actilyse®, Boehringer Ingelheim, Germany) in 2.5 ml saline. The animals in the fourth group (control) received sterile saline only at each time point. Those in the rtPA1 and rtPA2 groups received saline at the time points that they were not treated with rtPA.

Blood samples were taken at 6 and 24 h after inoculation by orbital puncture. Peritoneal fluid samples were taken after 24h via a laparotomy. After 5 days the animals were killed by CO_2 asphyxiation. The abdomen was inspected, with particular attention to the number and location of abscesses. All fibrin encased purulent collections were considered to represent abscesses. The presence of either diffuse purulent abdominal fluid, diffuse redness and edema of the peritoneum or diffuse adhesion of intra-abdominal organs was considered indicative of generalized peritonitis.

Animals

Male Wistar rats weighing 250-280 g (Harlan BV, Horst, the Netherlands) were housed two per cage

and accustomed to laboratory conditions for five days before the start of the experiment. All animals were weighed daily and had free access to water and standard rodent chow throughout the entire experimental period (Hope Farms, Woerden, the Netherlands). The Animal Ethics Review Committee of the Radboud University Nijmegen approved the study.

Sample collection

At 6 and 24 h after bacterial inoculation, 1 ml blood samples were taken by orbital plexus puncture, under anaesthesia, and collected in tubes containing 50 IU heparin for bacterial culturing. Peritoneal fluid samples were also collected under anaesthesia at 24 h after bacterial inoculation. After reopening the abdomen, 5 ml of sterile saline of 38°C was installed in the abdominal cavity. The abdomen was gently massaged and at least 2 ml of fluid was withdrawn. The samples were centrifuged for 10 min at 2000 rpm and supernatants stored at -80°C until further analysis.

Sample analysis

Heparinized blood samples (300 μl) were cultured for the presence of E. coli. Serial dilutions in Brain Heart Infusion broth were plated onto MacConkey blood agar plates. After 24 and 48 h of incubation at 37°C bacteria were identified and counted.

Tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β) and interleukin-10 (IL-10) were measured in peritoneal fluid. All assays were performed using commercially available ELISA kits (Endogen®, Pierce Biotechnology Inc, Rockford IL, USA). According to the manufacturer, the sensitivities for the various assays were < 10 pg/ml for TNF-α, < 12 pg/ml for IL-1β, < 16 pg/ml for IL-6 and < 3 pg/ml for IL-10, respectively. Cells were counted in peritoneal fluid after dilution of the sediment with saline (to a concentration of 50.000 cells/ml) and staining with May-Grunwald Giemsa reagent.

Statistics

Statistical analysis was performed using one-way ANOVA and Tukey Kramer multiple comparison test for multiple group comparison. Kruskal-Wallis and Mann-Whitney test were applied to process nonparametric data. Mortality rates were compared using a log rank test. Trend analysis for the parameters concerning abscess formation was performed using a Pearson chi square test and COX regression. P values < 0.05 were considered to be significant.

RESULTS

Abscesses

Recombinant t-PA treatment reduced abscess formation in animals which survived for 5 days (Figure 1A). The percentage of rats with abscesses gradually decreased from 88% in the untreated control group to 0% in the rtPA3 group. The mean number of abscesses per rat gradually decreased from 3.6 ± 2.7 (mean ± SD) in the controls to 0 in the rtPA3 group ($p = 0.003$, Figure 1B).
For both parameters, the percentage of rats with abcesses and the number of abcesses per rat, statistical analysis showed a significant trend for abscess reduction with the increasing number of rtPA doses given (Pearson Chi square, $p=0.004$ and COX regression, $p<0.0001$).

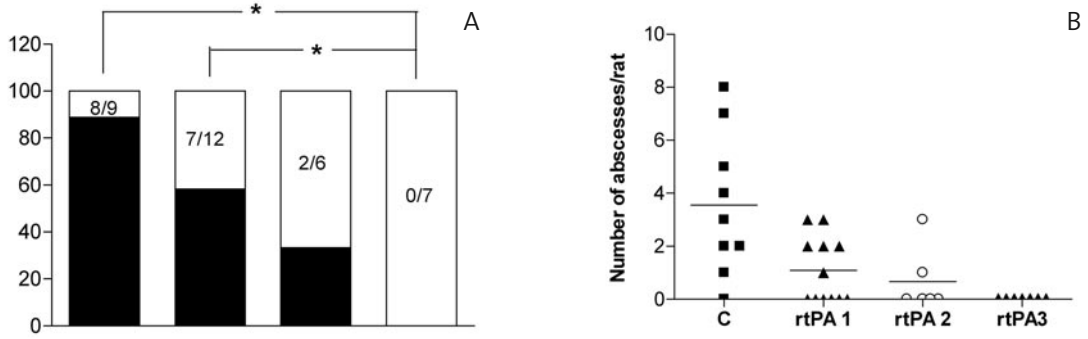

Figure 1a en 1b. **Abscess formation in surviving animals.**
Percentage of rats with abscesses (A) and mean number of abscesses per rat (B). * significant ($p<0.05$) difference between groups. The numbers within the columns in A indicate the actual number of rats with abscesses (represented as black bars) versus the total number of survivors in that group.

Blood cultures

Positive blood cultures (Table 1) were found in 50-79% of the rats after 6 h and in 73-90% of the rats after 24 h. No differences were found between control rats and rtPA treated rats, irrespective of the dose of rtPA given. Altogether, at 24 h the mean concentrations of E. coli were significantly ($p<0.05$) higher than at 6 h: 31.10^3 (range: 0.1-281 $.10^3$) cfu/ml and 275 (range: 40-1800) cfu/ml, respectively.

Group	% positive cultures		cfu/ml	cfu/ml
	6 h	24 h	6 h	24 h
C	79	82	200 (40-1000)	60.10^3 ($0.1\text{-}281.10^3$)
rtPA1	69	85	90 (40-740)	28.10^3 ($0.2\text{-}281.10^3$)
rtPA2	79	90	170 (60-1360)	22.10^3 ($1\text{-}185.10^3$)
rtPA3	50	73	640 (40-1800)	15.10^3 ($0.6\text{-}57.10^3$)

Table 1. **Blood cultures.**
Blood samples were cultured for the presence of E. coli at both 6 and 24 h after inoculation. Data pertain to the percentage (of all samples taken) which was positive for E. coli and to the median (and range) of the concentrations in the positive samples.

Cells and cytokines

One day after inoculation, cells in the abdominal fluid were predominantly neutrophils and, to a lesser extent, macrophages (Figure 2). The relative distribution over the various cell types remained unaffected by rtPA treatment. Also, the absolute numbers of cells were the same in the various groups (results not shown). Bacterial inoculation induced significant concentrations of various inflammatory cytokines in the abdominal fluid. Figure 3A-D shows the levels of respectively TNF-α, IL-1β, IL-6 and IL-10, as measured 24 h after inoculation. For none of these cytokines, treatment with any regimen of rtPA led to significant changes.

Clinical course and survival

All rats became severely ill after inoculation, as characterized by lack of movement, erect body hair and anorexia. Body weight decreased significantly ($p<0.05$) over the first 24 h after inoculation and stabilized thereafter. The changes in body weight during the experimental period (figure 4) were similar in the four groups. No bleeding complications were observed in any of the groups.
The overall survival rate was 62%. The rats that did not survive until the end of the experiment died mainly during the first 48 h after inoculation. The mortality rates of the experimental and control groups were similar. In all control rats with abscesses all three signs of generalized peritonitis were still present at autopsy 5 days after inoculation. In the rtPA treated rats with abscesses mostly just one sign of peritonitis was observed confined to one local area of inflammation.

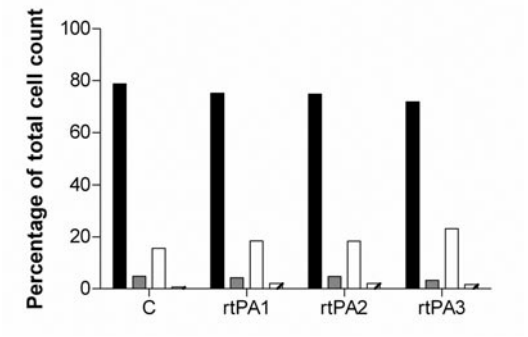

Figure 2. **Differential cell counts in abdominal fluid.**
Samples were taken at 24h after inoculation. Data on each cell type are expressed (mean + SD) as percentage of the total cell count. ▮ neutrophils, ▮ eosinophils, ▯ macrophage-type cells, ▨ lymphocytes.

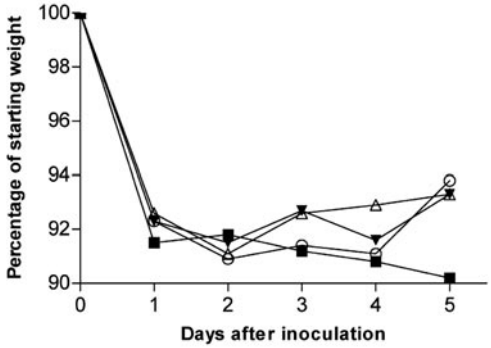

Figure 4.
Course of body weight during the experimental period after bacterial inoculation at day 0: ■, controls; ▼, rtPA1; ○, rtPA2; △, rtPA3.

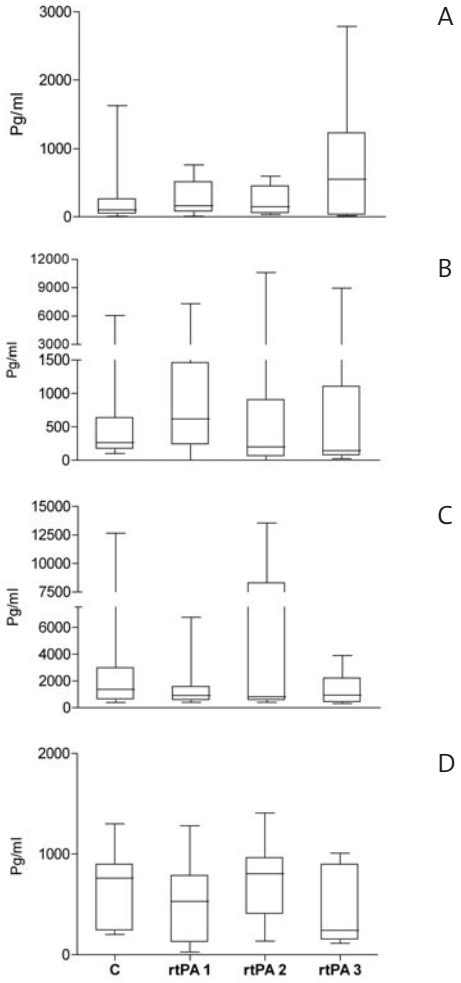

Figure 3. **Cytokines in abdominal fluid.**
Cytokine concentrations in pg/ml in peritoneal fluid collected at 24 after inoculation in animals surviving for 5 days. Data are depicted as medians with 25-75 percentiles in boxes and 5-95 percentiles represented as vertical lines. A = TNF-α, B = IL-1β, C = IL-6, D = IL-10

DISCUSSION

Intensifying fibrinolytic therapy after surgical debridement significantly improves the outcome in rats with secondary peritonitis without increasing morbidity or mortality. Intraperitoneal inoculation of a suspension of sterilized faeces with Escherichia coli (E. coli) and Bacteroides fragilis (B. fragilis) constitutes a valid animal model mimicking secondary peritonitis(21). Surgical debridement after 1 h provides a reproducible model to study residual abscesses after surgical treatment of generalized peritonitis(20). All animals from the control group displayed clear signs of a diffuse, generalized peritonitis at autopsy. The 1 h period between inoculation of the fecal suspension and surgical intervention is short, but a further delay results in an unacceptably high mortality when no antibiotics are administered(22). Although antibiotics are highly important as an adjunct to surgical therapy, we have chosen not to use antibiotics in the present experiment in order to study exclusively and selectively the potential of rtPA to act as an adjuvant to surgical therapy.

While hyaluronic acid and fungal polysaccharides have recently been shown to prevent abscess (and adhesion) formation in a rat peritonitis model, rtPA appears to be more effective(23;24). Rotstein and Kao studied the effect of a single dose of rtPA adminstered immediately after implantation of a a fibrin clot containing E. coli and B. fragilis(25). Our own group applied a single dose of rtPA in a slow release cellulose carrier immediately after surgical debridement for secondary peritonitis in a model which was similar to the one used in the present experiment(17). In both experimental conditions, where the same bacteria were inoculated but the timing of rtPA treatment was essentially different, a single application of a rtPA formula led to a reduction in abcess formation. The present experiment demonstrates that three doses rtPA in saline administered in the first 24 h after bacterial inoculation and surgical debridement are more effective than a single injection. Thus, the best effect of rtPA is achieved when treatment is given over, at least, the entire first 24 h after bacterial inoculation.

Adhesion formation was not an endpoint in this study. Therefore, adhesions were not actually scored or quantified. Yet, we did observe that in the vast majority of the rats treated with rtPA, the abdominal fluid was remarkably clear and the intra-abdominal organs were not adherent to each other while in the untreated rats adhesions occurred almost invariably.

RtPA is fibrin-specific(10) and has a half-life of 4-5 minutes in plasma (product information by Boeringer-Ingelheim). Yet the process of decreased fibrinolytic activity and increased coagulation in the abdominal compartment is considered to last during the course of peritonitis(26;27), but at least

for 12 h after inducing peritonitis in a rat model(28). Hence, a single dose of rtPA is not likely to completely reverse the disturbed balance between intra-abdominal fibrinolysis and coagulation.
The dose of rtPA applied in this study (approximately 4.1 mg/kg body weight for each dose given) has been chosen empirically, partly based on the results in prior experiments performed by van Goor et al.(17;29;30). The authors reported that coagulation activity rate in the rat is elevated by a factor 2.7 compared to man(29). It remains to be established how this dose compares to those given in clinical cases of peritonitis, where intraperitoneal doses of 0.5 and approximately 0.08 mg/kg body weight have been described in two case reports. In the first case rtPA was administered after repeat laparotomy in a neonate(15). The second report concerns the use of rtPA after a third episode of CAPD peritonitis(16). Although in these cases intra-abdominal fibrinolysis was pursued in a completely different situation, i.e. tertiary peritonitis, the data do suggest that lower doses than those used by us could be effective.
In the current study we observed no statistically differences in mortality between groups. This is in apparent contrast to both previous studies in rats with experimental abcesses or fecal peritonitis mentioned before(17;25), where a major concern has been that rtPA treatment was associated with significantly and seriously increased mortality. A possible explanation could be that rtPA induces an exaggerated local inflammatory (cellular and cytokine) or systemic response, but so far no data to this extent have been available. In animal models of bacterial peritonitis the inflammatory response has been characterized by highly elevated peritoneal levels of cytokines with a much lesser increase of systemic inflammatory mediators(31-33). On the cellular level, fecal peritonitis evokes an early influx of neutrophils followed by an influx of macrophages (34-36).
The peritoneal cytokine levels and the cell counts measured in the untreated control group of the present study are completely in line with expectations based on the data mentioned above. Moreover, since these parameters are similar in control and rtPA-treated animals, our data appear to exclude any significant effect of rtPA on local inflammation. Also, rtPA does not affect bacteremia. In addition, it is also noteworthy that neither local nor systemic bleeding complications, as a potentially major adverse effect of fibrinolytic therapy, have been observed. Systemic effects are unlikely in the first place since systemic uptake of rtPA after intraperitoneal delivery in the rat appears to be insignificant (30). In addition, the high systemic PAI levels, which have been described in peritonitis, will most likely counteract the systemic effects of rt-PA(30).
Thus, in the present model of fecal peritonitis, intra-abdominal application of rtPA over the first 24

h after surgical debridement is very effective in preventing the formation of intra-abdominal abcesses. The therapy is not associated with adverse events such as an increased local inflammatory response and/or increased mortality. We suggest that further research into its therapeutic efficacy and suitability in clinical application for the treatment of secondary peritonitis is warranted.

Acknowledgements
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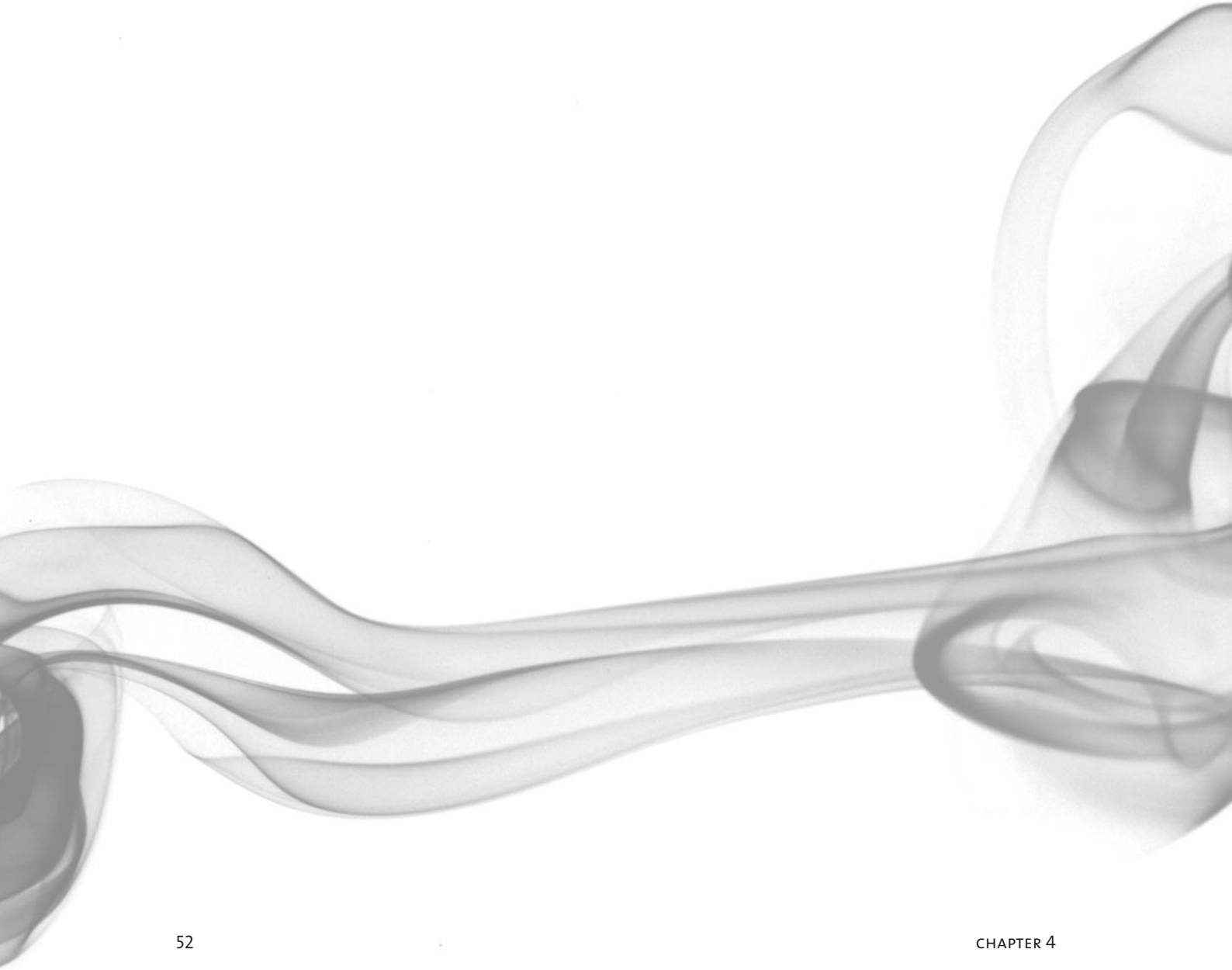
REFERENCE LIST

(1) Farthmann EH, Schoffel U. Epidemiology and pathophysiology of intraabdominal infections (IAI). Infection 1998 Sep;26(5):329-34.
(2) Bosscha K, van Vroonhoven TJ, van der Werken C. Surgical management of severe secondary peritonitis. Br J Surg 1999 Nov;86(11):1371-7.
(3) Marshall JC, Innes M. Intensive care unit management of intra-abdominal infection. Crit Care Med 2003 Aug;31(8):2228-37.
(4) Wong PF, Gilliam AD, Kumar S, Shenfine J, O'Dair GN, Leaper DJ. Antibiotic regimens for secondary peritonitis of gastrointestinal origin in adults. Cochrane Database Syst Rev 2005;(2):CD004539.
(5) Colizza S, Rossi S. Antibiotic prophylaxis and treatment of surgical abdominal sepsis. J Chemother 2001 Nov;13 Spec No 1(1):193-201.
(6) Schein M. Surgical management of intra-abdominal infection: is there any evidence? Langenbecks Arch Surg 2002 Apr;387(1):1-7.
(7) Ince A, Eroglu A, Tarhan O, Bulbul M. Peritoneal fibrinolytic activity in peritonitis. Am J Surg 2002 Jan;183(1):67-9.
(8) Reijnen MM, Bleichrodt RP, van Goor H. Pathophysiology of intra-abdominal adhesion and abscess formation, and the effect of hyaluronan. Br J Surg 2003 May;90(5):533-41.
(9) Holmdahl L, Falkenberg M, Ivarsson ML, Risberg B. Plasminogen activators and inhibitors in peritoneal tissue. APMIS 1997 Jan;105(1):25-30.
(10) Collen D, Lijnen HR. Tissue-type plasminogen activator: a historical perspective and personal account. J Thromb Haemost 2004 Apr;2(4):541-6.
(11) Singh V. Critical care assessment and management of acute ischemic stroke. J Vasc Interv Radiol 2004 Jan;15(1 Pt 2): S21-S27.
(12) Katchan BM. Thrombolytic therapy for pulmonary embolism. Can J Surg 2000 Dec;43(6):411-6.
(13) Beathard GA. Catheter thrombosis. Semin Dial 2001 Nov;14(6):441-5.
(14) Ray TL, Berkenbosch JW, Russo P, Tobias JD. Tissue plasminogen activator as an adjuvant therapy for pleural empyema in pediatric patients. J Intensive Care Med 2004 Jan;19(1):44-50.
(15) Diamond IR, Wales PW, Connolly B, Gerstle T. Tissue plasminogen activator for the treatment of intraabdominal abscesses in a neonate. Journal of Pediatric Surgery 2003 Aug;38(8):1234-6.
(16) Duch JM, Yee J. Successful use of recombinant tissue plasminogen activator in a patient with relapsing peritonitis. Am J Kidney Dis 2001 Jan;37(1):149-53.
(17) van Goor H, de Graaf JS, Kooi K, Sluiter WJ, Bom VJ, van der MJ, et al. Effect of recombinant tissue plasminogen activator on intra-abdominal abscess formation in rats with generalized peritonitis. J Am Coll Surg 1994 Oct;179(4):407-11.
(18) Rotstein OD, Kao J. Prevention of intra-abdominal abscesses by fibrinolysis using recombinant tissue plasminogen activator. J Infect Dis 1988 Oct;158(4):766-72.

(19) van Goor H, de Graaf JS, Kooi K, Bleichrodt RP. Gentamycin reduces bacteremia and mortality rates associated with the treatment of experimental peritonitis with recombinant tissue plasminogen activator. J Am Coll Surg 1995 Jul;181(1):38-42.
(20) Buyne OR, Bleichrodt RP, van Goor H, Verweij PE, Hendriks T. Tissue-type plasminogen activator prevents formation of intra-abdominal abscesses after surgical treatment of secondary peritonitis in a rat model. Int J Colorectal Dis 2006 Nov 7;.
(21) Parker SJ, Watkins PE. Experimental models of gram-negative sepsis. Br J Surg 2001 Jan;88(1):22-30.
(22) Rosman C, Bleichrodt RP, Wubbels GH, Sybesma T, Pasma P. The effect of intra-operative lavage with Taurolidine or Saline solution on mortality and plasma endotoxin level in rats with generalized peritonitis. Surg Res Comm 1990;(7):269-75.
(23) Reijnen MMPJ, Skrabut EM, Postma VA, Burns JW, van Goor H. Polyanionic Polysaccharides Reduce Intra-abdominal Adhesion and Abscess Formation in a Rat Peritonitis Model. Journal of Surgical Research 2001 Dec;101(2):248-53.
(24) Bae JS, Ahn SJ, Yim H, Jang KH, Jin HK. Prevention of intraperitoneal adhesions and abscesses by polysaccharides isolated from Phellinus spp in a rat peritonitis model. Ann Surg 2005 Mar;241(3):534-40.
(25) Rotstein OD, Kao J. Prevention of intra-abdominal abscesses by fibrinolysis using recombinant tissue plasminogen activator. J Infect Dis 1988 Oct;158(4):766-72.
(26) Holmdahl L, Eriksson E, al Jabreen M, Risberg B. Fibrinolysis in human peritoneum during operation. Surgery 1996 Jun;119(6):701-5.
(27) Holmdahl L. The role of fibrinolysis in adhesion formation. Eur J Surg Suppl 1997;(577):24-31.
(28) Vipond MN, Whawell SA, Thompson JN, Dudley HA. Effect of experimental peritonitis and ischaemia on peritoneal fibrinolytic activity. Eur J Surg 1994 Sep;160(9):471-7.
(29) van Goor H. In vitro studies on the effect of recombinant tissue plasminogen activator on the rat fibrinolytic system. Thesis: Fibrinolytic therapy in generalized peritonitis to prevent intraabdominal abscess formation. Nijmegen: 1996. p. 125-34.
(30) van Goor H, Bom VJ, van der MJ, Sluiter WJ, Geerards S, van der SW, et al. Pharmacokinetics of human recombinant tissue-type plasminogen activator, administered intra-abdominally, in a rat peritonitis model. Eur Surg Res 1996 Jul;28(4):287-94.
(31) Martineau L, Shek PN. Peritoneal cytokine concentrations and survival outcome in an experimental bacterial infusion model of peritonitis. Crit Care Med 2000 Mar;28(3):788-94.
(32) Montravers P, Maulin L, Mohler J, Carbon C. Microbiological and inflammatory effects of murine recombinant interleukin-10 in two models of polymicrobial peritonitis in rats. Infect Immun 1999 Apr;67(4):1579-84.
(33) Sewnath ME, Olszyna DP, Birjmohun R, ten Kate FJ, Gouma DJ, van der Poll T. IL-10-deficient mice demonstrate multiple organ failure and increased mortality during Escherichia coli peritonitis despite an accelerated bacterial clearance. J Immunol 2001 May 15;166(10):6323-31.
(34) Betjes MG, Tuk CW, Visser CE, Zemel D, Krediet RT, Arisz L, et al. Analysis of the peritoneal cellular immune system during CAPD shortly before a clinical peritonitis. Nephrol Dial Transplant 1994;9(6):684-92.

(35) Yao V, McCauley R, Cooper D, Platell C, Hall JC. Peritoneal Mesothelial Cells Produce Cytokines in a Murine Model of Peritonitis. Surgical Infections 2004;5(3):229-36.
(36) Broche F, Tellado JM. Defense mechanisms of the peritoneal cavity. Curr Opin Crit Care 2001 Apr;7(2):105-16.



Chapter 4

Both tissue-type plasminogen activator and urokinase prevent intra-abdominal abscess formation after surgical treatment of peritonitis in the rat

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ABSTRACT

Background

Prevention of intra-abdominal abscess formation constitutes an important goal in treatment of secondary peritonitis. Fibrinolytic therapy may be effective in this respect. The efficacy of recombinant tissue plasminogen activator (rtPA) and urokinase is compared in a preclinical model for surgical treatment of peritonitis.

Methods

Peritonitis was induced by intraperitoneal bacterial challenge in male Wistar rats. One hour afterwards, surgery was performed. Four groups (n=20) were treated with either rtPA, urokinase, streptokinase (a negative protein control) or saline. Blood cultures were taken at 6 and 24 h; cell counts and cytokine measurements were performed in peritoneal fluid at 1, 3 and 5 days. After 5 days, animals were killed and intraperitoneal abscess formation was analyzed.

Results

Both rtPA and urokinase strongly (by > 75%) and significantly ($p<0.05$) reduced abscess formation without negative side effects. No bleeding complications were observed. Fibrinolytic therapy altered the intraperitoneal cellular distribution (less neutrophils and more macrophages) but did not essentially alter courses of interleukin-6 and interleukin-10 (decreasing in time) or TNF-α (increasing in time) levels.

Conclusion

Both rtPA and urokinase effectively and safely reduce abscess formation in a rat model for treatment of secondary peritonitis. Fibrinolytic therapy should be further developed for clinical application.

INTRODUCTION

Secondary peritonitis results from contamination of the abdominal cavity with gastro-intestinal contents, in the majority of cases caused by perforation of the gastro-intestinal tract. Despite optimal treatment, invariably including surgical intervention, organ-supportive care and antibiotic therapy, peritonitis remains an important cause of morbidity and mortality in surgical practice and in the intensive care unit(1-3). One of the sequelae of peritonitis is the formation of intra-abdominal abscesses. Drainage is the preferred treatment and can be performed either surgical or percutaneously. By either means, abscess drainage will be successful in approximately 70% of all cases (4). However, surgical treatment i.e. reexploration by laparotomy, contributes to increased short- and long-term morbidity (5). In addition, abscesses recur in 10% of treated cases(6). Therefore, prevention of abscess formation is an important goal in the search for the optimal treatment of generalized peritonitis.
Intra-abdominal abscesses originate from contaminated fibrin clots. Fibrin deposition occurs in the abdominal cavity as a result of a disturbed balance between fibrinogenesis and fibrinolysis, due to mesothelial cell damage. Bacteria caught in fibrin clots cannot be reached by antibiotics or the immunologic defence systems in the abdominal cavity and thus form the nidus for intra-abdominal abscess formation. Fibrinolytic therapy may therefore be an attractive adjuvant therapy to diminish the risk of abscess formation.
There is little information about the clinical application of fibrinolytics in peritonitis. Thrombolytic agents administered intraperitoneally appear to facilitate antibiotic penetration into the bio film formed by certain bacteria (7;8). Numerous case reports of intraperitoneal thrombolytic adjunctive therapy for recurrent or persistent peritoneal dialysis-associated peritonitis have indicated that these agents may have a role in the treatment of selected patients(8;9). Next to tissue-type plasminogen activator (tPA), the fibrinolytic enzymes urokinase and streptokinase are widely used in clinical practice to install thrombolytic therapy(10-12).
Earlier preclinical studies by us(13) and others(14) have indeed indicated the potential of exogenous tPA to reduce intraperitoneal abscess formation during generalized peritonitis. Recently, we have optimized a model in the rat to study abscess formation after induced intra-abdominal infection(15) and initiated a series of experiments aiming to establish that fibrinolytic therapy can be conducted effectively and safely. In a dose-finding study of limited size, using small groups of rats, we have first established 'proof of principle', by demonstrating that repeated intraperitoneal injection of human

recombinant tPA (rtPA) can lead to a significant reduction of abscess formation(16). The present experiment focuses on the efficacy and safety of two differently acting fibrinolytic drugs in a model which shares important, but not all, features with the clinical (surgical) treatment of secondary peritonitis. For instance, antibiotics were deliberately not given at the present stage. We hypothesized that both rtPA and urokinase can be used intraperitoneally to reduce abscess formation without inducing potential side-effects such as bacteriaemia, mortality and bleeding complications.

MATERIALS AND METHODS

Experimental design

Eighty rats were randomly divided into four groups of twenty rats. Peritonitis was induced in all rats by intraperitoneal injection of 2 ml of a faecal suspension containing 10^4 cfu Bacteroides fragilis (strain 25285 from the American Type Culture Collection) and 10^5 cfu Escherichia coli (strain 25922 from the American Type Culture Collection), respectively(16) . One hour after inoculation surgery was performed. The rats were anesthetized by inhalation of a mixture of isoflurane-oxygen-nitrous oxide. The abdominal cavity was debrided including partial resection of macroscopically polluted omentum and rinsed with 5 ml of normal saline of 38°C. The abdomen was closed with a running suture of 4.0 Polyglactin 910 (Vicryl®, Ethicon, Amersfoort, The Netherlands), the skin was closed with staples. Rats received 5 ml normal saline subcutaneously for resuscitation.

In two experimental groups, either rtPA (Actilyse®, Boehringer Ingelheim, Germany) in a dose of 1.25 mg in 2.5 ml saline or Urokinase (Urokinase®, MEDAC, Hamburg) in a dose of 725.000 IU in 2.5 ml saline was installed in the abdomen at the end of surgery (1h), at 6 h via percutaneous injection and at 24 h via relaparotomy. At these same time points the two control groups received either 2.5 ml of normal saline or streptokinase (Streptase®, Aventis Pharma BV, Hoevelaken) in a dose of 725.000 IU in 2.5 ml saline. The streptokinase group has been added as a negative protein control since streptokinase does not affect the rat coagulatory system(17).

After 5 days the animals were killed by CO_2 asphyxiation. The abdomen was inspected, with particular attention to the number and location of abscesses as well as to signs of intra-abdominal bleeding. All fibrin-encased purulent collections were considered to represent abscesses. The presence of diffuse purulent abdominal fluid, diffuse redness, and edema of the peritoneum was considered indicative of generalized peritonitis. The investigator was blinded for the therapy given.

Animals

Male Wistar rats weighing 250-280 g (Harlan BV, Horst, the Netherlands) were housed two per cage and accustomed to laboratory conditions for five days before the start of the experiment. All animals were weighed daily and had free access to water and standard rodent chow throughout the entire experimental period (Hope Farms, Woerden, the Netherlands). The Animal Ethics Review Committee of the Radboud University Nijmegen approved the study.

Sample collection

Six and 24 h after inducing peritonitis, 300 µl blood samples were taken from all rats by orbital plexus puncture under general anesthesia, and collected in tubes containing 50 IU heparin for bacterial culturing. Peritoneal fluid samples were collected from all animals under general anesthesia, at 24, 72 and 120 h after bacterial inoculation as follows: after removing two skin staples and one abdominal wall stitch to gain access, 5 ml of sterile saline of 38°C was installed in the abdominal cavity and at least 2 ml of fluid were withdrawn. The samples were centrifuged for 10 min at 750 x g and supernatants stored at -80°C.

Sample analysis

Heparinized blood samples (300 µl) were cultured for the presence of E. coli. Serial dilutions in Brain Heart Infusion broth were plated onto MacConkey blood agar plates. After 24 and 48 h of incubation at 37°C bacteria were identified and counted. Levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-10 (IL-10) were measured in peritoneal fluid by using commercially available ELISA kits (Endogen®, Pierce Biotechnology Inc, Rockford IL, USA). According to the manufacturer, the sensitivities for the assays were <10 pg/ml for TNF-α, <16 pg/ml for IL-6 and <3 pg/ml for IL-10, respectively. Cells were counted in peritoneal fluid after dilution of the sediment with saline (to a concentration of 50.000 cells/ml) and staining with May-Grunwald Giemsa reagent.

Statistics

Statistical analysis was performed using one-way ANOVA and Tukey-Kramer multiple comparison test for parametric data, while a Kruskal-Wallis followed by Dunn's multiple comparison test were applied to process nonparametric data. Trend analysis for the parameters concerning abscess formation was performed using a Chi-square test and COX regression. P values < 0.05 were considered significant.

RESULTS

Survival and clinical course

Two out of 80 animals died within 48 hours after inducing peritonitis, both in the urokinase group. Septic shock was believed to be the probable cause of death since no abdominal cause could be identified. No further mortality was observed during the study. Altogether, there was no significant difference between groups regarding mortality.

All rats became severely ill after inoculation, as characterized by lack of movement, pilo-erection and anorexia. Body weight decreased during the first 24 h after inoculation and stabilized thereafter. The changes in body weight during the experimental period (Figure 1) were similar in the four groups. Almost invariably, all signs of generalized peritonitis were observed at post mortem examination, both in untreated rats and those treated with streptokinase. No bleeding complications were observed in any of the animals.

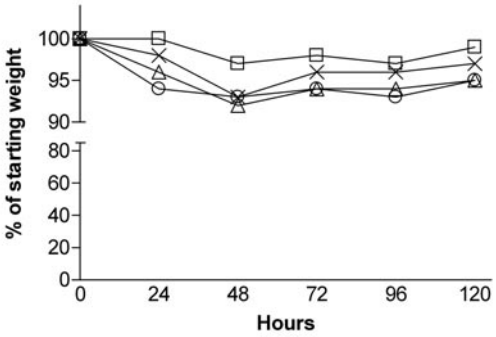

Figure 1. **Blood cultures at 24 hours after inoculation.**
Course of body weight during the experimental period after bacterial inoculation at day 0. ○: controls; □: streptokinase; △: rtPA; ×: urokinase.

Abscesses

Both rtPA and urokinase treatment significantly reduced abscesses formation as compared to the controls and the streptokinase treated group. The percentage of surviving rats with abscesses was significantly lower in the groups treated with rtPA and urokinase than in both control and streptokinase groups: 55 % vs. 100 % ($p<0.0001$, Chi-square test) (Figure 2A).

The mean number (± SD) of abscesses per rat was1.4 ±1.9 and 0.9 ± 1.1 in the rtPA and urokinase group versus 4.9 ± 1.9 and 5.6 ± 2.3 in the control and streptokinase group, respectively (Figure 2B). The

number of rats with abscesses in the diaphragmatic region was significantly lower in the rtPA and urokinase groups than in the control and streptokinase groups (Table 1): 3 and 4 versus 17 en 19, respectively (p<0.0001, Chi-square). The same was noted, to a lesser extent, in the left paracolic region: 4 and 1 versus 10 and 6, respectively (p<0.01, Chi-square).

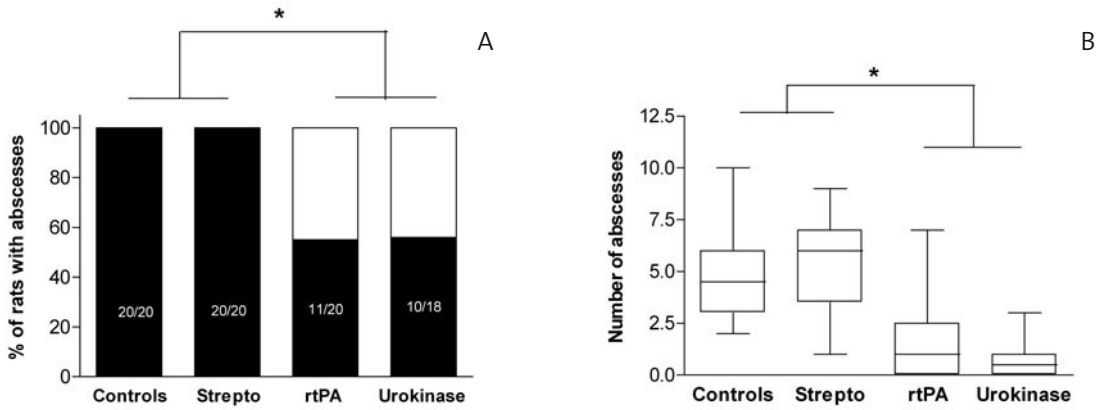

Figure 2a en 2b. **Abscess formation in surviving animals.**
Percentage of surviving rats with abscesses (A) and mean (+ SD) number of abscesses per rat (B). The numbers within the columns in A indicate the actual number of rats with abscesses (represented as black bars) versus the total number of survivors in that group. Data are depicted as medians with 25-75 percentiles in boxes and 5-95 percentiles represented as vertical lines *: significant (p<0.05, A: Chi-square test; B: Kruskal-Wallis plus Dunn's test) difference between groups.

	n	Diaphragm	Left paracolic	Right paracolic
Controls	20	17	10	14
Streptokinase	20	19	6	18
rtPA	20	3*	4	10
Urokinase	18	4*	1**	8

Table 1. **Distribution of abscesses.**
Each column displays the number of rats in each group with abscesses in one of the three locations, where abscess formation was observed most frequently. Some rats had abscesses at multiple locations. * p<0.05 (Chi-square test) vs both controls and streptokinase groups. ** p<0.05 vs controls.

Blood cultures

Positive blood cultures were found in 40-50% of all rats at 6 h after inoculation and this frequency was significantly increased within each group at 24 h ($p<0.05$, Chi-square, Table 2). There were no significant differences between the groups at either time point.

There was a large variation between animals regarding the concentration of E. coli and no significant differences between the groups (Figure 3). The median concentration of cultured E. coli cfu/ml, (Table 2) was significantly ($p< 0.05$) higher in the surviving animals at 24 h than at 6 h, in all groups except the streptokinase group.

Group	% positive cultures		cfu/ml	cfu/ml
	6 h	24 h	6 h	24 h
Controls	40	74	220 (20-24.10^3)	570 (20-78.10^3)
Streptokinase	50	85	2360 (20-17.10^3)	660 (20-67.10v)
rtPA	45	80	300 (20-11.10^3)	680 (20-70.10^3)
Urokinase	40	63	100 (20-11.10^3)	160 (20-67.10^3)

Table 2. **Blood cultures.**
Blood samples were cultured for the presence of E. coli at both 6 and 24 h after inoculation. Data pertain to the percentage (of all samples taken), which was positive for E. coli and to the median (and range) of the concentrations in the positive samples.

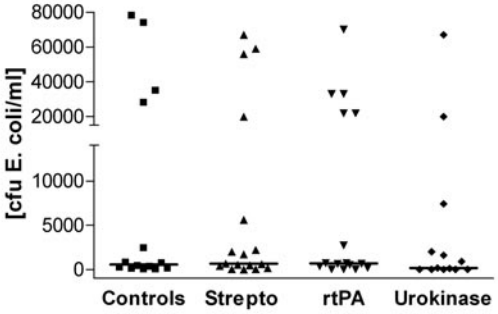

Figure 3. **Blood cultures at 24 hours after inoculation.**
Each symbol represents a concentration of bacteria expressed as number of colony forming units per ml. Horizontal lines indicate median values in each group.

Cells and cytokines

Since streptokinase treatment had no effect whatsoever on abscess formation, which was the primary outcome parameter in this study, quantification of cell numbers and cytokine concentrations was only performed in the two experimental groups and the saline control group.

For all groups, the absolute number of cells counted in the peritoneal fluid declined after 72 h. However, it did not differ between groups at any time point. Differential cell count of peritoneal fluid showed predominantly neutrophils at 24 h. Thereafter neutrophil numbers decreased, while the relative presence of the other cell types increased. Generally speaking, fibrinolytic therapy resulted in less neutrophils and lymphocytes and more macrophages and eosinophils in time (Figure 4).

Significant ($p<0.05$, ANOVA plus Tukey-Kramer) differences between the three groups were only encountered in neutrophil and macrophage counts. Neutrophil counts in the controls were higher than in the rtPA group at 24 h and higher than in both experimental groups at 72 and 120 h. The same trend was observed for lymphocytes. Contrarily, macrophage counts in the control group remained significantly below those in the rtPA group at 24 and 72 h and below those in both experimental groups at 120 h. The same was observed for eosinophils but not significantly so. No significant differences whatsoever were found between the rtPA and urokinase groups

For all groups, peritoneal fluid IL-6 and IL-10 concentrations were highest at 24 h and lowest at 120 h (Figure 5). At 24 h, these cytokine levels ranged widely, upper levels reaching nearly 5000 and 1700 pg/ml for IL-6 and IL-10, respectively. At none of the time points investigated did IL-6 concentrations differ significantly between the three groups. For IL-10, there were no differences at 24 h, but at both 72 and 120 h levels in the control group remained significantly ($p<0.05$, Kruskal-Wallis and Dunn's multiple comparison test) higher than in the urokinase group.

On the average, bacterial inoculation led to measurable TNF-α concentrations 24 h later, which were similar in all groups. In contrast to the interleukins, TNF-α increased with time and levels at 120 h were significantly higher in all groups. At this time, concentrations in the rtPA group were significantly lower than those in the control group.

In order to investigate possible mediators which contribute to abscess formation, we compared the cytokine levels and cellular counts in all rats (from control and both experimental groups) which showed abscesses and those that were free of abscesses at autopsy. At none of the time points significant differences were found.

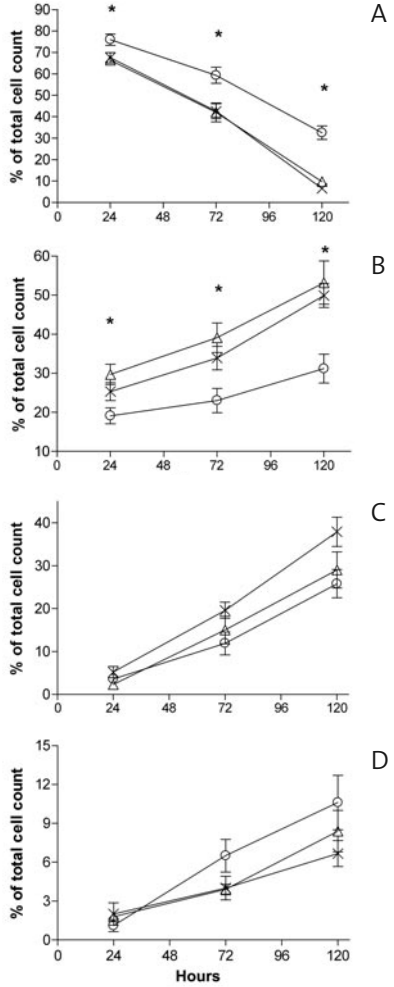

Figure 4. **Differential cell counts in abdominal fluid.**
A: neutrophils, B: macrophages, C: eosinophils, D: lymphocytes. Data on each cell type are expressed (n=10, mean + SD) as percentage of the total cell count, ○: controls; △: rtPA; ×: urokinase. *: significant ($p<0.05$, ANOVA) difference between groups.

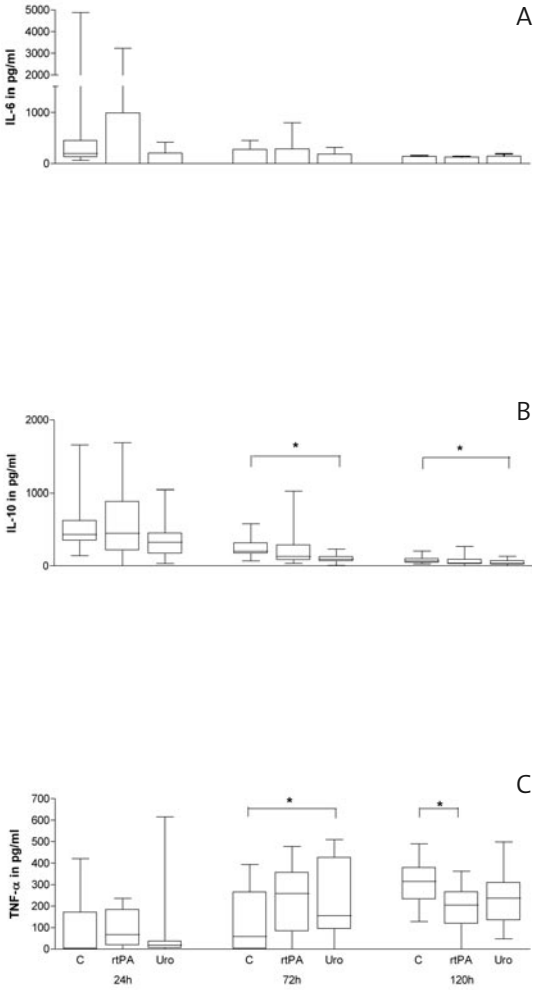

Figure 5. **Cytokines in abdominal fluid.**
Data for IL-6 (A), IL-10 (B) and TNF-α (C) are depicted as medians (measurements in all surviving animals) with 25-75 percentiles in boxes and 5-95 percentiles represented as vertical lines. *: significant ($p<0.05$, Kruskal-Wallis plus Dunn's test) difference between groups.

DISCUSSION

Intra-abdominal application of rtPA or urokinase significantly reduces the number of abscesses in the abdominal cavity in rats with generalized peritonitis. The streptokinase group has been added as a negative protein control since streptokinase does not affect the rat coagulatory system(17). The fact that this group behaves similarly to the saline controls supports the conclusion that the effect of both exogenous rtPA and urokinase is a specific one.

The animal model used, is a modification of a well-established procedure(15;16) to induce generalized peritonitis. It consistently reflects several aspects of peritonitis as observed in the clinical situation with transiently elevated cytokine levels and typical changes in the cellular composition of the peritoneal fluid. After surgical intervention, signs of local and generalized peritonitis remain present for an extended period and rats will almost invariably develop intra-abdominal abscesses. These abscesses are typically found in the sub diaphragmatic and paracolic spaces. It should be emphasized that the ultimate goal of our series of experiments is to optimize the treatment of generalized peritonitis by intra-abdominal application of fibrinolytics. As yet, we do not purport to present a model that reflects the clinical situation of peritonitis in all its aspects. Here, we focus on prevention of abscess formation and thus mortality is not an essential feature of the model(15). In addition, although we recognize antibiotic therapy to be highly important as an adjunct to surgery, we have chosen to not (yet) use antibiotics in order to assess exclusively and selectively the potential of fibrinolytics to act as an adjuvant to surgical therapy. Still, antibiotics are especially helpful in treating the systemic effects of peritonitis, while, to our knowledge, a beneficial effect on abscess formation has not been documented. In a pilot study we have established that a regimen of metronidazole and ceftriaxone does not reduce abscess formation in our rat model (unpublished data).

The two-edged effect of fibrin formation has been recognized already in the early 1980s. On one hand, fibrin prevents dissemination of bacteria throughout the abdominal cavity. On the other hand, fibrin protects bacteria from the immunologic defenses, thus forming a nidus for abscess formation and ongoing intra-abdominal sepsis(18;19). Performing surgery itself reduces peritoneal tPA activity and thus can be a trigger for fibrin formation in the abdominal cavity(20;21). The present model comprises repeated laparotomies, essential to collect samples of peritoneal fluid. By limiting the size of the laparotomy as much as possible we have tried to limit its influence. A factual investigation of their effect falls outside the scope of the present experiment. Since the procedure is performed in all

groups, the outcome of the present experiment will not be affected.
One could easily imagine fibrinolytic therapy to affect wound healing, and certainly so in the intestine, although the existing literature(22-24) appears to be largely reassuring. Preliminary experiments in our laboratory confirm that anastomotic healing is not affected under non-infectious conditions.
Since fibrin formation is essential to the formation of both surgical adhesions and abscesses, therapy directed at its prevention is thought to be potentially beneficial in the prevention of both complications. However, anti-adhesive agents do not necessarily have any effect on abscess formation(25-27).
The potential usefulness of tPA has been suggested in the past, but its application mode used in those peritonitis models has led to seriously enhanced mortality (13;14). This has been hypothesized to be caused by a massive release of bacteria from infected fibrin clots, causing bacteremia, a modulated immune response or a combination of both. Bacterial cultures from intra-abdominal abscesses confirmed the local presence of bacteria, yet bacteremia could not be confirmed(13). In the present experiment, fibrinolytic therapy does lead to neither mortality nor increased bacteremia.
The typical course in cellular response to abdominal inflammation and infection(28) is observed: neutrophils are gradually replaced by macrophages and eosinophils. In both rtPA and urokinase groups, a lower neutrophil (and lymphocyte) count together with a higher macrophage (and eosinophil) count is observed with time, indicating a, quantitative rather than qualitative, modulation of the local cellular response. Recent experiments, investigating the behaviour of tPA -/- mice in intra-abdominal sepsis, also suggest that there may be a role for tPA in the inflammatory cell recruitment in the peritoneal cavity(29). The effects of fibrinolytic therapy on the peritoneal cytokine response are also limited and quantitative rather than qualitative. The course of the IL-6 levels in all groups in this study is as to be expected in peritonitis and is consistent with experimental data(16;30-32) that describe an elevation of IL-6 and TNF-α. Recent clinical data(33) report a significant elevation of Il-6, IL-10 and TNF-α at the onset of disease with normalization at the 5th day. Altogether, the data on the local inflammatory response do not suggest any major effect of rtPA or urokinase.
Recent experimental data support the thesis that manipulation of the intra-abdominal fibrinolytic capacity can affect the course of disease in a cecal ligation and puncture model for polymicrobial peritonitis. Intra-abdominal application of rtPA(34) results in an improved bacterial clearance but no improvement of survival, while peritoneal lavage with activated protein C also may rebalance coagulation and fibrinolysis within compartments and improve survival(35). In addition, a reduced local inflammatory response and improved survival have been observed after intra-abdominal

treatment with recombinant human antithrombin(36).
The clinical use of fibrinolytics is associated with bleeding complications in- or outside the operative field(37;38). In septic rats, systemic bleeding is unlikely because plasma levels of Plasminogen Activator Inhibitor (PAI) are elevated, consequently leading to the forming of inactive tPA-PAI complexes(39) In addition, previous studies confirm that total plasminogen activity after intra-abdominal application of tPA is very low(40). Indeed, no bleeding complications are seen in any of the rats from the present study, either in the rtPA group or in the urokinase group.
The doses of urokinase and rtPA are based on their use in the clinical setting for treatment of thrombo-embolic disorders(37;38), taking into consideration that the coagulation activity rate in the rat is elevated by a factor 2.7 compared to man(17). In addition, the dose of rtPA as applied in the current study has already proven its value in earlier studies(13;40). The recent dose finding study has demonstrated that repeat doses at 1, 6 and 24 h yield the best results with respect to abscess reduction(16).
The use of urokinase in stead of rtPA may be advantageous. Firstly, the half-life of urokinase is fourfold the half-life of rtPA. A longer-lasting effect may be advantageous in topical treatment if it lessens the number of doses needed to serve its purpose. However, further studies are needed to prove this to be true since urokinase is less fibrin-specific than rtPA(38). Secondly, urokinase is less expensive than rtPA. Our results prove both fibrinolytics to be equally effective, although the use of urokinase would lead to a 35% reduction of costs as compared to rtPA. As yet, there are no data on a possible connection between dose and outcome parameters such as abscess reduction, morbidity and mortality.
In order to further determine optimal conditions for fibrinolytic therapy, the effects of lower doses of these drugs and their effectiveness after prolonged or delayed administration will be investigated, as well as their efficacy in the presence of antibiotics. We believe fibrinolytic agents to be very promising for clinical use in the treatment of secondary peritonitis.

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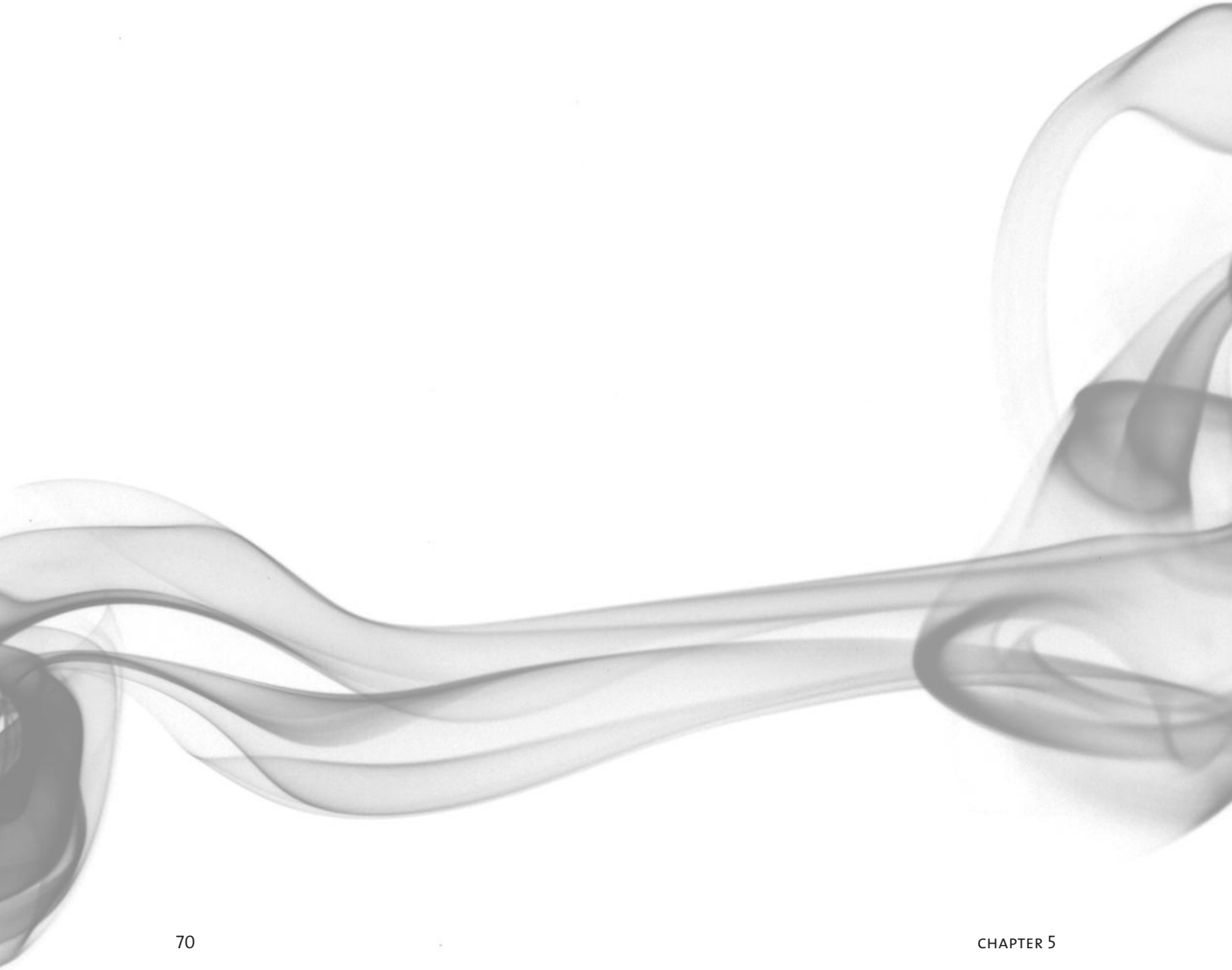
REFERENCE LIST

(1) Marshall JC, Innes M. Intensive care unit management of intra-abdominal infection. Crit Care Med 2003 Aug;31(8):2228-37.
(2) Schein M. Surgical management of intra-abdominal infection: is there any evidence? Langenbecks Arch Surg 2002 Apr;387(1):1-7.
(3) Bosscha K, van Vroonhoven TJ, van der Werken C. Surgical management of severe secondary peritonitis. Br J Surg 1999 Nov;86(11):1371-7.
(4) Sirinek KR. Diagnosis and treatment of intra-abdominal abscesses. Surg Infect (Larchmt) 2000;1(1):31-8.
(5) Rosin D, Zmora O, Khaikin M, Bar ZB, Ayalon A, Shabtai M. Laparoscopic management of surgical complications after a recent laparotomy. Surg Endosc 2004 Jun;18(6):994-6.
(6) Tomita H, Osada S, Miya K, Matsuo M. Delayed recurrence of postoperative intra-abdominal abscess: an unusual case and review of the literature. Surg Infect (Larchmt) 2006 Dec;7(6):551-4.
(7) Stewart PS. Mechanisms of antibiotic resistance in bacterial biofilms. Int J Med Microbiol 2002 Jul;292(2):107-13.
(8) Worland MA, Radabaugh RS, Mueller BA. Intraperitoneal thrombolytic therapy for peritoneal dialysis-associated peritonitis. Ann Pharmacother 1998 Nov;32(11):1216-20.
(9) Duch JM, Yee J. Successful use of recombinant tissue plasminogen activator in a patient with relapsing peritonitis. Am J Kidney Dis 2001 Jan;37(1):149-53.
(10) El-Menyar AA, Altamimi OM, Gomaa MM, Dabdoob W, Abbas AA, bdel Rahman MO, et al. Clinical and biochemical predictors affect the choice and the short-term outcomes of different thrombolytic agents in acute myocardial infarction. Coron Artery Dis 2006 Aug;17(5):431-7.
(11) Mullen MT, McGarvey ML, Kasner SE. Safety and efficacy of thrombolytic therapy in postoperative cerebral infarctions. Neurol Clin 2006 Nov;24(4):783-93.
(12) Ueshima S, Matsuo O. Development of new fibrinolytic agents. Curr Pharm Des 2006;12(7):849-57.
(13) van Goor H, de Graaf JS, Kooi K, Sluiter WJ, Bom VJ, van der MJ, et al. Effect of recombinant tissue plasminogen activator on intra-abdominal abscess formation in rats with generalized peritonitis. J Am Coll Surg 1994 Oct;179 (4):407-11.
(14) Rotstein OD, Kao J. Prevention of intra-abdominal abscesses by fibrinolysis using recombinant tissue plasminogen activator. J Infect Dis 1988 Oct;158(4):766-72.
(15) Buyne OR, Bleichrodt RP, Verweij PE, Groenewoud HM, van Goor H, Hendriks T. A peritonitis model with low mortality and persisting intra-abdominal abscesses. Int J Exp Pathol 2006 Oct;87(5):361-8.
(16) Buyne OR, Bleichrodt RP, van Goor H, Verweij PE, Hendriks T. Tissue-type plasminogen activator prevents formation of intra-abdominal abscesses after surgical treatment of secondary peritonitis in a rat model. Int J Colorectal Dis 2007 Jul;22(7):819-25.

(17) van Goor H. In vitro studies on the effect of recombinant tissue plasminogen activator on the rat fibrinolytic system. Thesis: Fibrinolytic therapy in generalized peritonitis to prevent intraabdominal abscess formation. Nijmegen: 1996. p. 125-34.
(18) Ahrenholz DH, Simmons RL. Fibrin in peritonitis. I. Beneficial and adverse effects of fibrin in experimental E. coli peritonitis. Surgery 1980 Jul;88(1):41-7.
(19) Rotstein OD. Role of fibrin deposition in the pathogenesis of intraabdominal infection. Eur J Clin Microbiol Infect Dis 1992 Nov;11(11):1064-8.
(20) Holmdahl L. The role of fibrinolysis in adhesion formation. Eur J Surg Suppl 1997;(577):24-31.
(21) Neudecker J, Junghans T, Ziemer S, Raue W, Schwenk W. Effect of laparoscopic and conventional colorectal resection on peritoneal fibrinolytic capacity: a prospective randomized clinical trial. Int J Colorectal Dis 2002 Nov;17(6):426-9.
(22) Houston KA, McRitchie DI, Rotstein OD. Tissue plasminogen activator reverses the deleterious effect of infection on colonic wound healing. Ann Surg 1990 Feb;211(2):130-5.
(23) Evans DM, McAree K, Guyton DP, Hawkins N, Stakleff K. Dose dependency and wound healing aspects of the use of tissue plasminogen activator in the prevention of intra-abdominal adhesions. Am J Surg 1993 Feb;165(2):229-32.
(24) Buckenmaier CC, III, Summers MA, Hetz SP. Effect of the antiadhesive treatments, carboxymethylcellulose combined with recombinant tissue plasminogen activator and Seprafilm, on bowel anastomosis in the rat. Am Surg 2000 Nov;66(11):1041-5.
(25) Bedirli A, Gokahmetoglu S, Sakrak O, Ersoz N, Ayangil D, Esin H. Prevention of intraperitoneal adhesion formation using beta-glucan after ileocolic anastomosis in a rat bacterial peritonitis model. Am J Surg 2003 Apr;185(4):339-43.
(26) Sikkink CJ, de MB, Bleichrodt RP, van GH. Auto-cross-linked hyaluronic acid gel does not reduce intra-abdominal adhesions or abscess formation in a rat model of peritonitis. J Surg Res 2006 Dec;136(2):255-9.
(27) Muller SA, Treutner KH, Haase G, Kinzel S, Tietze L, Schumpelick V. Effect of intraperitoneal antiadhesive fluids in a rat peritonitis model. Arch Surg 2003 Mar;138(3):286-90.
(28) Broche F, Tellado JM. Defense mechanisms of the peritoneal cavity. Curr Opin Crit Care 2001 Apr;7(2):105-16.
(29) Renckens R, Roelofs JJ, Florquin S, de Vos AF, Pater JM, Lijnen HR, et al. Endogenous tissue-type plasminogen activator is protective during Escherichia coli-induced abdominal sepsis in mice. J Immunol 2006 Jul 15;177(2):1189-96.
(30) Martineau L, Shek PN. Peritoneal cytokine concentrations and survival outcome in an experimental bacterial infusion model of peritonitis. Crit Care Med 2000 Mar;28(3):788-94.
(31) Montravers P, Maulin L, Mohler J, Carbon C. Microbiological and inflammatory effects of murine recombinant interleukin-10 in two models of polymicrobial peritonitis in rats. Infect Immun 1999 Apr;67(4):1579-84.
(32) Sewnath ME, Olszyna DP, Birjmohun R, ten Kate FJ, Gouma DJ, van der Poll T. IL-10-deficient mice demonstrate multiple organ failure and increased mortality during Escherichia coli peritonitis despite an accelerated bacterial clearance. J Immunol 2001 May 15;166(10):6323-31.
(33) Haecker FM, Fasler-Kan E, Manasse C, Fowler B, Hertel R, von SD. Peritonitis in childhood: clinical relevance of cytokines in the peritoneal exudate. Eur J Pediatr Surg 2006 Apr;16(2):94-9.

(34) van Veen SQ, Meijers JC, Levi M, van Gulik TM, Boermeester MA. Effects of intra-abdominal administration of recombinant tissue plasminogen activator on coagulation, fibrinolysis and inflammatory responses in experimental polymicrobial peritonitis. Shock 2007 May;27(5):534-41.
(35) van Veen SQ, Levi M, van Vliet AK, Florquin S, van Gulik TM, Boermeester MA. Peritoneal lavage with activated protein C alters compartmentalized coagulation and fibrinolysis and improves survival in polymicrobial peritonitis. Crit Care Med 2006 Nov;34(11):2799-805.
(36) van Veen SQ, Cheung CW, Meijers JC, van Gulik TM, Boermeester MA. Anticoagulant and anti-inflammatory effects after peritoneal lavage with antithrombin in experimental polymicrobial peritonitis. J Thromb Haemost 2006 Nov;4(11):2343-51.
(37) Bell WR. Present-day thrombolytic therapy: therapeutic agents--pharmacokinetics and pharmacodynamics. Rev Cardiovasc Med 2002;3 Suppl 2:S34-S44.
(38) Deitcher SR JMR. Pharmacologic and clinical characteristics of thrombolytic agents. Rev Cardiovasc Med 2003 Mar;2(Suppl.):S25-S33.
(39) Ince A, Eroglu A, Tarhan O, Bulbul M. Peritoneal fibrinolytic activity in peritonitis. Am J Surg 2002 Jan;183(1):67-9.
(40) van Goor H, Bom VJ, van der MJ, Sluiter WJ, Geerards S, van der SW, et al. Pharmacokinetics of human recombinant tissue-type plasminogen activator, administered intra-abdominally, in a rat peritonitis model. Eur Surg Res 1996 Jul;28(4):287-94.



Chapter 5

Timing and dose of tissue plasminogen activator to prevent abscess formation after surgical treatment of secondary peritonitis in the rat

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Submitted

ABSTRACT

Background

Early administration of fibrinolytics after surgical treatment of peritonitis in the rat reduces abscess formation. In clinical practice surgical treatment can be delayed for several reasons and there is concern about side effects of post-operative administration of fibrinolytics. The current study investigates the effect of delayed and prolonged treatment with intraperitoneal recombinant tissue plasminogen activator (rtPA) and the effect of lowering the dose of rtPA.

Study Design

Peritonitis was induced via intra-abdominal injection of a feces/bacteria mixture in male Wistar rats. Surgical debridement was performed after 1h. In the first experiment four groups of animals were treated with 1.25 mg of rtPA starting at different time points, one group receiving prolonged treatment. In a second experiment two groups were treated with either 0.25 mg or 1.25 mg of rtPA. Abdominal fluid samples were taken at 24, 72 and 120 h for IL-6, IL-10 and TNF-α measurements and cell counts. After 5 days the abdomen was inspected for abscesses.

Results

Early administration of rtPA in both doses significantly reduced the number of rats with abscesses and the abscess load per rat as compared to untreated controls. Delayed treatment significantly reduced abscess load but not the incidence of abscesses. No adverse side effects were observed and no meaningful differences in the local inflammatory response were found. RtPA was most effective when applied early and continued for 72 h, although mortality increased after prolonged treatment.

Conclusions

RtPA consistently reduces intra-abdominal abscess formation. Future clinical research is warranted addressing the issues of dosage and the potential benefit of early and prolonged administration of rtPA.

INTRODUCTION

Secondary peritonitis is a life threatening condition mostly occurring after disruption of the integrity of the gastrointestinal tract(1). In the last 30 years, mortality rates have hardly improved despite important advancements in perioperative care, antibiotic treatment and surgical therapy(2-4). Recurrent or ongoing abdominal sepsis is recognized as a main determinant of outcome(5). Intra-abdominal abscesses are an important source of abdominal infection and cannot always be treated successfully by either surgical or percutaneous techniques(6;7). Prevention of abscess formation after surgery for peritonitis therefore remains an essential target for research.

Since fibrin deposition is an intrinsic element in abscess formation it is logical to investigate the potential of fibrinolytic therapy in this respect. Indeed, earlier preclinical studies have indicated that exogenous tissue-type plasminogen activator (tPA) may reduce intraperitoneal abscess formation during generalized peritonitis, though at the cost of significant mortality(8;9). Recently, we have optimized a model which is suitable to study the mechanisms involved in, and prevention of, intra-abdominal abscess formation after surgical treatment of generalized peritonitis(10). Further studies have demonstrated that repeated intraperitoneal administration of human recombinant tPA (rtPA) significantly reduces abscess formation without consistent serious side-effects(11;12). The model shares important, but not all, features with the clinical situation. For instance, contrary to clinical practice, antibiotics were deliberately not given in order to selectively study the potential beneficial effects of fibrinolytics. Also, rtPA treatment has been restricted to the first 24 hours after bacterial inoculation and surgical debridement. In the clinical setting, early administration of rtPA might not always be feasible: the time between onset of disease and presentation of clinical symptoms can vary, establishing the diagnosis can be delayed and hence the time of initiating adequate therapy will vary(13). Therefore we have addressed the following questions related to the rtPA regimen chosen so far: is prolonged treatment more effective than treatment during 24 hours and can delayed rtPA treatment still be effective in reducing abscess formation? Anticipating the need to maximally reduce the treatment-related risk profile, the efficacy of lowering the dose was also investigated.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 250-280 g (Harlan BV, Horst, the Netherlands) were housed two per cage and accustomed to laboratory conditions for five days before the start of the experiment. All animals had free access to water and standard rodent chow throughout the entire experimental period (Hope Farms, Woerden, the Netherlands). The clinical condition and weight of the rats were monitored daily. The Animal Ethics Review Committee of the Radboud University Nijmegen approved the study.

Induction and treatment of peritonitis

Peritonitis was induced in all rats by intraperitoneal injection of 2 ml of a fecal suspension containing 10^4 cfu Bacteroides fragilis (strain 25285 from the American Type Culture Collection) and 10^5 cfu Escherichia coli (strain 25922 from the American Type Culture Collection), respectively(10).
One hour after inoculation surgical therapy was performed. The rats were anesthetized by inhalation of a mixture of isoflurane-oxygen-nitrous oxide. The abdominal cavity was debrided including partial resection of macroscopic polluted omentum and rinsed with 5 ml of sterile saline of 38°C. The abdomen was closed with a running suture of 4.0 Polyglactin 910 (Vicryl ®, Ethicon, Amersfoort, The Netherlands), the skin was closed with staples. Rats received 5 ml normal saline subcutaneously for resuscitation.

Experiment 1

One hundred animals were randomly divided into five groups of 20 rats each. The first group (rtPA) was treated with 1.25 mg of rtPA (Actilyse®, Boehringer Ingelheim, Germany) dissolved in 2.5 ml saline, which was left in the abdomen at the end of the laparotomy at 1 hour. Via percutaneous injection additional doses rtPA were given at 6 and 24 hours. The second group (rtPA-long) received prolonged rtPA treatment: doses of rtPA were given at 1, 6, 24, 36, 48, 60 and 72 hours. The third group (rtPA-24) received three doses of 1.25 mg rtPA in sterile saline from one day after bacterial inoculation, at 24, 30 and 48 hours. In the fourth group (rtPA-48), rtPA treatment was delayed for 2 days and doses were given at 48, 54 and 72 hours. Finally, the control group was treated with sterile saline at 1, 6 and 24 hours after the induction of peritonitis (also see table 1).

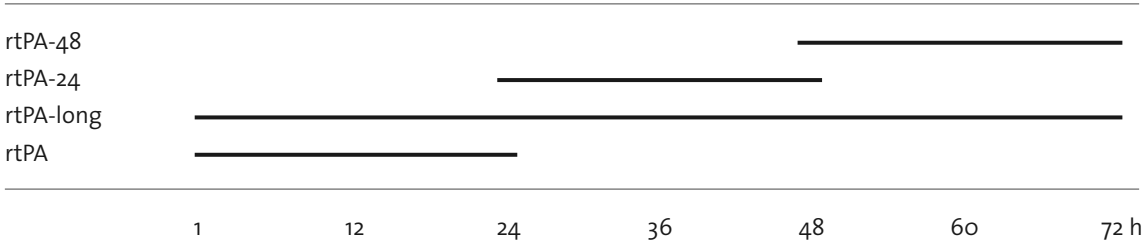

Table 1. **Treatment scheme, experiment 1.** Duration of rtPA administration displayed as a horizontal line.

After five days the surviving rats were killed by CO_2 asphyxiation. The abdomen was inspected, with particular attention to signs of generalized peritonitis, the number and location of abscesses and signs of intra-abdominal bleeding. All fibrin-encased purulent collections were considered abscesses. The abscess load was represented by an abscess score. To this end, all abscesses were classified semi-quantitatively according to their diameter: < 1 mm: 1; 1 - 2 mm: 2 and > 2 mm: 3 points. For each rat, the sum of these points represented the abscess score. Generalized peritonitis was defined as the presence of either diffuse purulent abdominal fluid, diffuse redness and edema of the peritoneum or diffuse fibrinous adhesions between intra-abdominal organs.

Experiment 2

Forty five rats were randomly divided into three groups of 15 rats each. Two groups were treated similar to the control and rtPA group described in the preceding experiment. In the third group (rtPA-low) each dose of rtPA was lowered five-fold to 0.25 mg in 2.5 ml saline. Further procedures were as described above.

Sample collection and analysis

In all animals peritoneal fluid samples were collected under anesthesia, at 24 and 72 h after bacterial inoculation. After removing two skin staples and one abdominal wall stitch to gain access, 5 ml of sterile saline of 38°C was installed in the abdominal cavity. The abdomen was gently massaged and at least 2 ml of fluid were withdrawn. The samples were centrifuged for 10 min at 750 x g and supernatants stored at -80° C until further analysis.

Levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-10 (IL-10) were measured in peritoneal fluid by using commercially available ELISA kits (Endogen®, Pierce Biotechnology Inc, Rockford IL, USA). According to the manufacturer, the sensitivities for the various assays were < 10 pg/ml for TNF-α, < 16 pg/ml for IL-6 and < 3 pg/ml for IL-10, respectively.
The cellular content of peritoneal fluid samples was assessed after dilution of the sediment with saline (to a concentration of 50.000 cells/ml) and staining with May-Grunwald Giemsa reagent.

Statistics
Mortality rates were compared by log rank test. Since the main objective of this study was to establish the effect of the different treatments per se, the functional outcome parameters in each of the four experimental groups were tested for significance in relation to the control group. To correct for the fact that multiple comparisons were made, pair-wise comparisons were done with a two-tailed Mann-Whitney test using a level of significance of $2\alpha/k$ where k is the total number of pair wise comparisons. For instance, comparison of the four experimental groups with the control group in experiment 1 yields a significant difference if $p<0.025$. Trend analysis for the parameters regarding abscess formation was performed using a Pearson chi square test and COX regression.

RESULTS

Experiment 1
Survival and clinical course
All rats became severely ill after inoculation, as characterized by lack of movement, pilo-erection and anorexia. Body weight decreased during the first 24 h after inoculation and stabilized thereafter. The changes in body weight during the experimental period were similar in all groups. The overall survival rate was 68%. Twenty-three of the 32 rats that died prematurely did so within 48 h after inoculation. Survival was lowest in the rtPA-long group: 45% vs 90% in the control group ($p = 0.002$). The other survival rates did not differ significantly from the controls and were 60%, 75% and 70% in the rtPA-, rtPA-24- and rtPA-48-groups, respectively. No bleeding complications were observed in rats that either survived until the end of the study or that died prematurely. In all surviving rats two or more signs of peritonitis were observed at post mortem examination. In 2 animals that died, massive fibrinous adhesions and purulent collections were observed in the abdomen.

Abscesses

Only when rtPA treatment was initiated immediately after surgical debridement, the percentage of surviving animals with abscesses was significantly reduced, from 89% in the control group to 58% and 33% in the rtPA and rtPA-long groups, respectively (Figure 1A). All rtPA treatments reduced both the number of abscesses per rat (Figure 1B) and the abscess score (Figure 1C). For both parameters, the median values in the rtPA, rtPA-long, rtPA-24 and rtPA-48 groups were significantly below those in the control group.

Comparing the location of abscesses, significantly more rats with abscesses at the diaphragm and in the left and right paracolic gutter were observed in the control group as compared to the rtPA-long group. Significantly more abscesses in the left paracolic gutter were found in controls as compared to the rtPA group, $p<0.05$ (Table 2).

	n	Diaphragm	Left paracolic	Right paracolic
Controls	18	12	14	10
rtPA	12	4	5*	4
rtPA-long	9	2*	1*	0*
rtPA-24	15	3	11**	5
rtPA-48	14	6	7	4

Table 2. **Distribution of abscesses in experiment 1.**
Each column displays the number of rats in each group with abscesses in one of the three locations, where abscess formation was observed most frequently. Some rats had abscesses at multiple locations. * $p<0.05$ (Chi-square test) vs controls. **$p<0.05$ vs RTPA-long

Cytokines

There were no differences between controls and any of the experimental groups. Values at 24 h are not shown since treatment of some groups was started at that time point or later. Between 24 and 72 hours, levels of IL-6 and IL-10, but not TNF-α, decreased in all groups. At 72 hours (Figure 2) median IL-10 levels in the rtpa-24 group were reduced by 50% in comparison with controls: this was the only significant ($p = 0.016$) difference found between experimental and control groups.

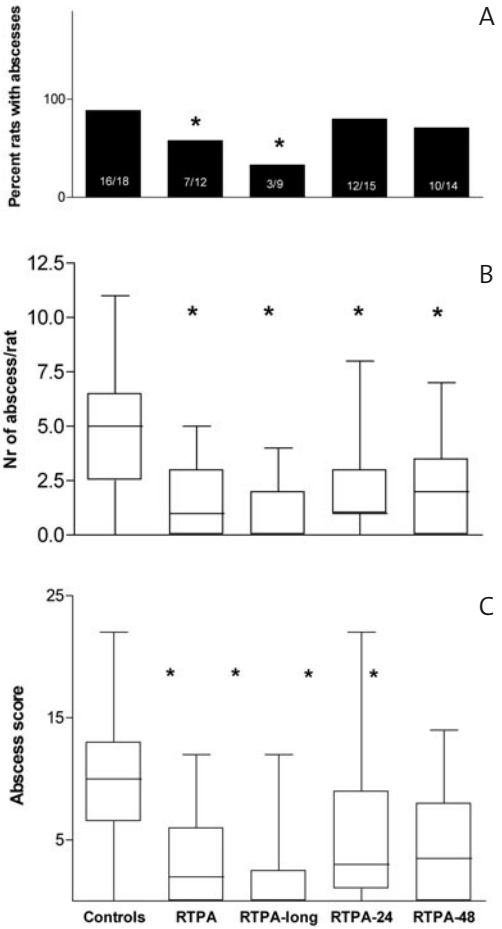

Figure 1a, 1b, 1c. **Abscess formation in experiment 1.**
Percentage of rats with abscesses (A), number of abscesses per rat (B) as well as the total abscess score per rat (C) are given. In A, numbers within bars give the actual number of (surviving) rats within each group with abscesses. In B and C, data are depicted as medians with 25-75 percentiles in boxes and 5-95 percentiles represented as vertical lines. *: $p < 0.05$ vs untreated controls.

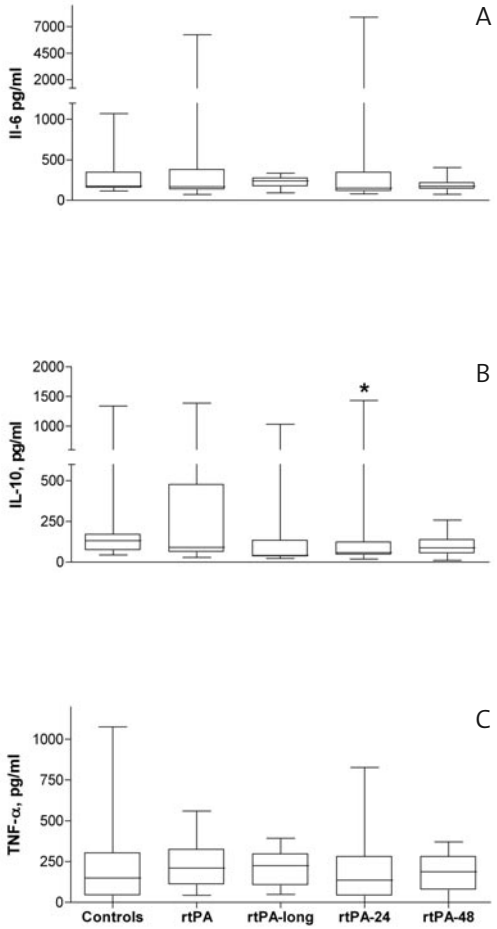

Figure 2. **Cytokines in peritoneal fluid.**
IL-6 (A) , IL-10 (B) and TNF-α (C) concentrations (pg/ml) in peritoneal fluid collected at 72 h after inoculation. Data are depicted as medians with 25-75 percentiles in boxes and 5-95 percentiles represented as vertical lines. *: $p < 0.05$ vs untreated controls.

Cells

The total cell numbers in peritoneal samples did not significantly differ between groups. The percentage of neutrophils declined significantly with time (from 79% at 24 h to 18 % at 120 h). Eosinophils and macrophages increased from 4 to 24% and from 16 to 45%, respectively, while lymphocyte counts remained relatively stable (Figure 3).

Experiment 2

Survival and clinical course

As in experiment 1 all rats showed signs of severe illness. Sixteen rats died prematurely, 10 within the first 48 h. The overall survival rate was 64%. Survival rates were 60%, 87% and 47% for controls, rtPA-low and rtPA groups, respectively, the difference between the latter two groups reaching significance ($p = 0.017$). No bleeding complications were found. In all surviving rats two or more signs of peritonitis were observed. At autopsy an intraperitoneal hematoma was observed in 3 (all rtPA group) out of 16 rats that died prematurely.

Abscesses

The percentage of surviving rats with abscesses was significantly ($p = 0.03$) reduced in both the rtPA and the rtPA-low groups (Figure 4A). The median (range) number of abscesses per rat was significantly ($p<0.05$) higher in controls than in both the rtPA and rtPA-low groups (Figure 4B).

Cytokines and cells

Between 24 and 72 hours, levels of IL-6 and IL-10 decreased significantly in controls and rtPA groups and TNF-α levels increased in the rtPA-low group.

At 24 h levels of IL-6 and IL-10 were significantly ($p<0.05$) lower in the rtPA-low group than in the rtPA and control groups while TNF-α levels showed no differences. At 72 h levels of cytokines did not significantly differ between groups.

The total cell numbers in peritoneal samples were similar in all groups at all time points. Qualitative changes were the same as in experiment 1 and there was no clear and sustained effect of rtPA. If any, the increase in the percentage of eosinophils seemed somewhat stronger after rtPA treatment, but only between 24 and 72 hours.

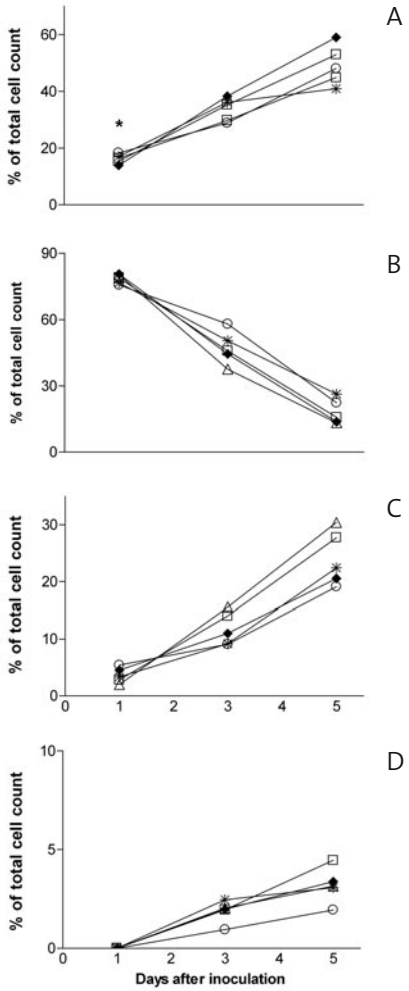

Figure 3. **Differential cell counts in abdominal fluid.**
Samples were taken at 24, 72 and 120 h after inoculation. A: macrophages, B: neutrophils, C: eosinophils, D: lymphocytes. Data on each cell type are expressed as mean percentage of the total cell count.○: controls;□: rtpPA;△: rtPA-long;◆: rtPA-24;×: rtPA-48.*: p<0.05 for rtPA-24 vs untreated controls.

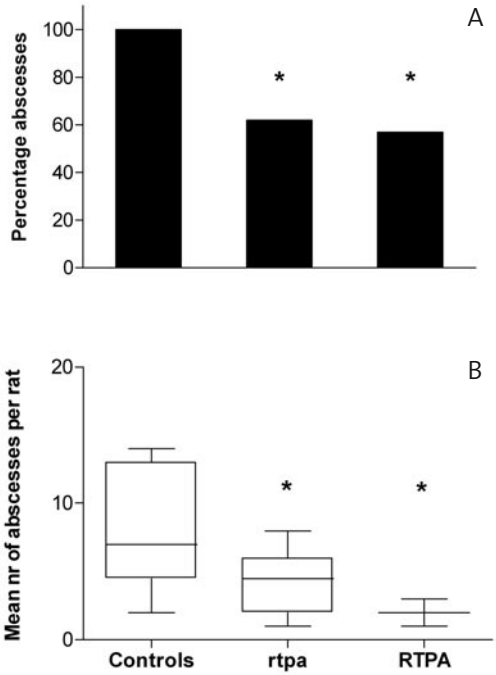

Figure 4a, 4b. **Abscesses formation in experiment 2.**
Percentage of rats with abscesses (A) and number of abscesses per rat (B). In B data are depicted as medians with 25-75 percentiles in boxes and 5-95 percentiles represented as vertical lines. *: p < 0.05 vs untreated controls.

DISCUSSION

Intraperitoneal application of rtPA consistently reduces intra-abdominal abscess formation after (surgical) treatment of secondary peritonitis. The beneficial effects of rtPA are reflected not only by reduction of the number of rats with abscesses, but also by the number of abscesses per rat. The latter is still significantly reduced when intra-abdominal fibrinolytic treatment is delayed for 24 h, although then the incidence of intra-abdominal abscesses is not affected. Reduction of the abscess load, expressed as the abscess score can even be achieved until 48 hours after onset of disease.

In the current model the animals do not die from the abscesses. Presumably, mortality is caused by systemic sepsis and multi organ failure which develop early. Generally speaking, infection models are well known for their significant variations in the course of disease between experiments. In our model, animals are very sick during the first 24 h and their survival may depend on conditions particular to each experiment, for instance, the particular batch of animals used or the batch of inoculum, although obviously full efforts are made to standardize the latter. Such variance could explain the observed difference in mortality between the control groups from (the independent) experiments 1 and 2.

One could comment on the fact that mortality in the rtPA treated rats tends to be higher than in the controls. In the present model, this is accepted as a calculated risk. Activation of coagulation and formation of fibrin deposits as a result of inflammation are considered instrumental in containing inflammatory activity to the site of injury or infection. Bacteria caught in fibrin clots are released much faster into the abdominal cavity when intra-abdominal fibrinolytics are given, provoking the risk of bacteremia, sepsis and death. This experimental set up has been chosen deliberately, in order to optimally study the specific potential of rtPA in preventing abscess formation. Therefore, mortality is not a primary outcome parameter. Administration of systemic antibiotics in this experimental model cancels the differences between rtPA treated rats and controls in this respect(14;15)Although systemic antibiotics do decrease mortality, they do not prevent abscess formation(15;16). It should be emphasized that antibiotics are vital in the treatment of patients with generalized peritonitis and thus will always be part of therapy in clinical practice.

The dose of 1.25 mg of rtPA for each gift has been chosen according to results in clinical studies on thrombo-embolic diseases, taking into account that rat coagulatory activity is 2.7 times higher than in humans(17). Applying a five-fold lower dose of rtPA is still effective in significantly reducing

abscess formation. One of the concerns of applying a fibrinolytic drug in peritonitis is the risk of bleeding complications. The chance of local bleeding complications is considered to be low in a rat peritonitis model. This is explained by a decreased plasminogen activator activity (PAA), probably as a result of elevated levels of plasminogen activator inhibitor (PAI)(18). Dose reduction may have no clear advantage over the chosen dosing scheme under standardized conditions, but might be beneficial in specific high-risk clinical cases. For instance, in patients suffering ongoing low-grade abdominal sepsis who are at risk of developing recurrent abscesses(19). In these patients prolonged fibrinolytic therapy in a low dose may reduce the need for surgical (re)intervention for abscesses. This is of clinical importance for surgical reintervention itself bears a 22-77% mortality rate(20).

In the current study prolonged treatment with rtPA tends to yield the best results with respect to abscess reduction, yet at the expense of significantly higher mortality as compared to controls. Therefore, if prolonged application of rtPA would seem advantageous, particular attention should be paid to dosage.

Until now the results of our preceding studies revealed no adverse effects of rtPA(11;12), if treatment was given as in the current rtPA groups. The increased mortality rate after prolonged treatment, as observed in experiment 1, cannot be explained in terms of bleeding complications or an altered local inflammatory response. The number of bleeding complications in the group that received prolonged treatment was nil. Although an immune modulatory effect of rtPA has been suggested recently(21), neither significant difference in the intraperitoneal cellular response, nor differences in local cytokine expression during the first 72 hours after inoculation were observed. Both cellular and cytokine response are most pronounced during the first 48 to 72 hours after the onset of peritonitis(22;23). Presumably, increased mortality is a logical consequence of premature release of bacteria into the abdominal cavity (see above) and not related to interference of rtPA with the immunological defense mechanisms in the abdominal cavity.

When interpreting the results of the current study one should realize that peritonitis was induced in a standardized way and was treated at a fixed time point. The finding that rtPA still seems to be effective in terms of abscess reduction if started 24 h, and possibly even 48 h after bacterial inoculation is of particular interest. In the clinical setting, abdominal sepsis may present itself in different ways and it is known for its broad variety in causes and severity of the infection(24;25). Patient's as well as doctor's delay account for variations in the timing of adequate therapy(26). Immediate application of fibrinolytics at the end of the operation in patients with intra-abdominal sepsis seems

to be the most logical strategy to prevent intra-abdominal abscess formation.
Altogether, these results point out the potential benefits of applying rtPA as an adjunct to the standard treatment of peritonitis in the clinical setting. Obviously, safe rtPA dosages, possibly derived from those used in for instance thrombo-embolic diseases(27;28), should be tested in a phase II study. Bearing in mind that frequently the clinical course will be protracted requiring peritonitis patients to receive supportive treatment for a longer period, the effects of prolonged treatment with a low dose of rtPA should be investigated.

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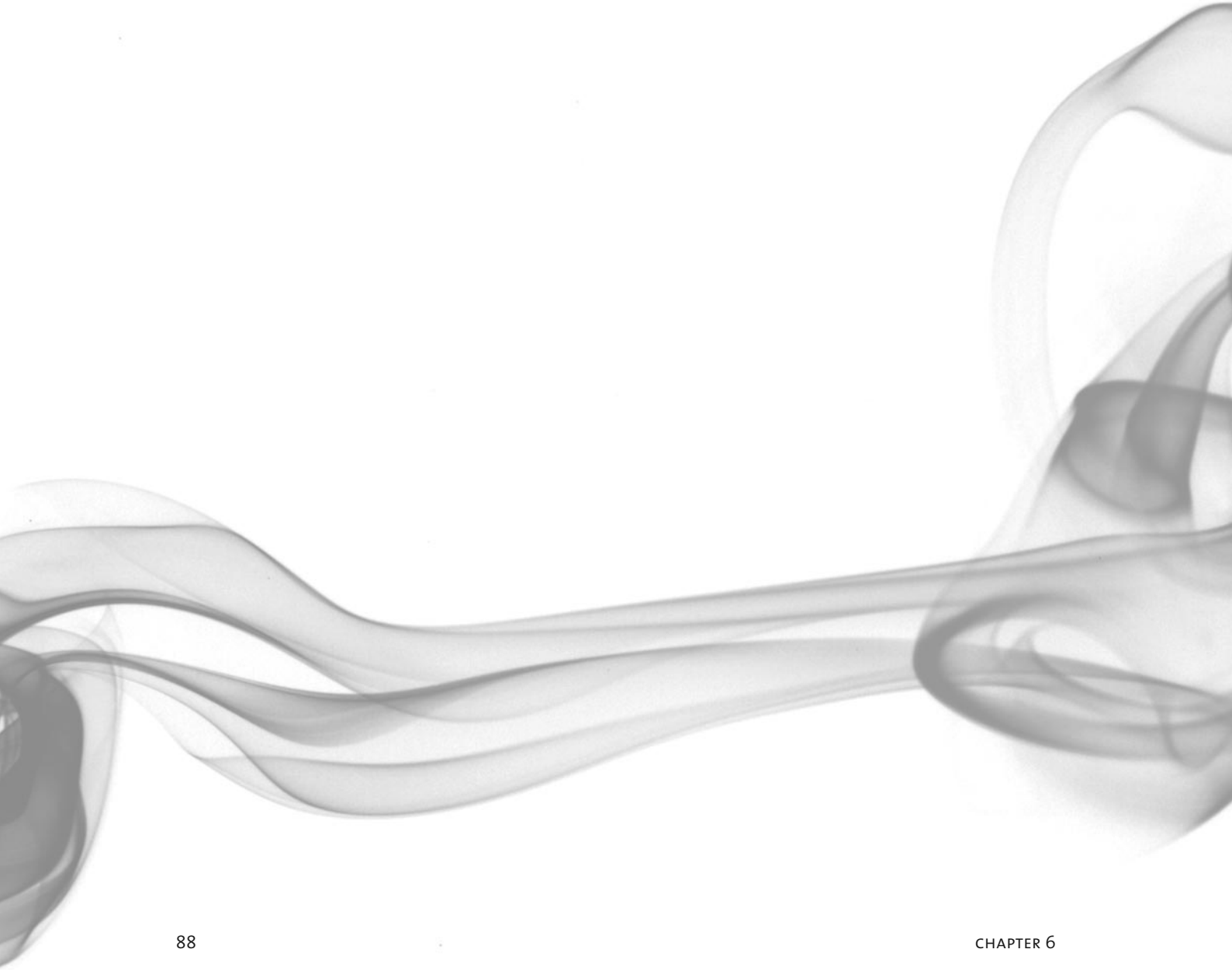
REFERENCE LIST

(1) Boermeester MA. Surgical approaches to peritonitis. Br J Surg 2007 Nov;94(11):1317-8.
(2) Chong AJ, Dellinger EP. Current treatment of intraabdominal infections. Surg Technol Int 2005;14:29-33.
(3) Lamme B, Boermeester MA, Belt EJ, van Till JW, Gouma DJ, Obertop H. Mortality and morbidity of planned relaparotomy versus relaparotomy on demand for secondary peritonitis. Br J Surg 2004 Aug;91(8):1046-54.
(4) Schein M. Surgical management of intra-abdominal infection: is there any evidence? Langenbecks Arch Surg 2002 Apr;387(1):1-7.
(5) Marshall JC, Innes M. Intensive care unit management of intra-abdominal infection. Crit Care Med 2003 Aug;31(8):2228-37.
(6) Marshall JC. Intra-abdominal infections. Microbes Infect 2004 Sep;6(11):1015-25.
(7) Sirinek KR. Diagnosis and treatment of intra-abdominal abscesses. Surg Infect (Larchmt) 2000;1(1):31-8.
(8) Rotstein OD, Kao J. Prevention of intra-abdominal abscesses by fibrinolysis using recombinant tissue plasminogen activator. J Infect Dis 1988 Oct;158(4):766-72.
(9) van Goor H, de Graaf JS, Kooi K, Sluiter WJ, Bom VJ, van der MJ, et al. Effect of recombinant tissue plasminogen activator on intra-abdominal abscess formation in rats with generalized peritonitis. J Am Coll Surg 1994 Oct;179(4):407-11.
(10) Buyne OR, Bleichrodt RP, Verweij PE, Groenewoud HM, van Goor H., Hendriks T. A peritonitis model with low mortality and persisting intra-abdominal abscesses. Int J Exp Pathol 2006 Oct;87(5):361-8.
(11) Buyne OR, Bleichrodt RP, van Goor H., Verweij PE, Hendriks T. Tissue-type plasminogen activator prevents formation of intra-abdominal abscesses after surgical treatment of secondary peritonitis in a rat model. Int J Colorectal Dis 2007 Jul;22(7):819-25.
(12) Buyne OR, van Goor H, Bleichrodt RP, Verweij PE, Hendriks T. Both tissue-type plasminogen activator and urokinase prevent intraabdominal abscess formation after surgical treatment of peritonitis in the rat. Surgery 2008 Jul;144(1):66-73.
(13) Hartl WH, Wolf H, Schneider CP, Kuchenhoff H, Jauch KW. Secular trends in mortality associated with new therapeutic strategies in surgical critical illness. Am J Surg 2007 Oct;194(4):535-41.
(14) van Goor H, de Graaf JS, Kooi K, Bleichrodt RP. Gentamycin reduces bacteremia and mortality rates associated with the treatment of experimental peritonitis with recombinant tissue plasminogen activator. J Am Coll Surg 1995 Jul;181(1):38-42.
(15) Buyne OR, Bleichrodt RP, van Goor H, Verweij PE, Hendriks T. Plasminogen activator, but not systemic antibiotic therapy, prevents abscess formation in an experimental model of secondary peritonitis. Br J Surg 2008 Oct;95(10):1287-93.
(16) Hau T, Nishikawa RA, Phuangsab A. The effect of bacterial trapping by fibrin on the efficacy of systemic antibiotics in experimental peritonitis. Surg Gynecol Obstet 1983 Sep;157(3):252-6.

(17) van Goor H. In vitro studies on the effect of recombinant tissue plasminogen activator on the rat fibrinolytic system. Thesis: Fibrinolytic therapy in generalized peritonitis to prevent intraabdominal abscess formation. Nijmegen: 1996. p. 125-34.
(18) van Goor H, Bom VJ, van der MJ, Sluiter WJ, Geerards S, van der SW, et al. Pharmacokinetics of human recombinant tissue-type plasminogen activator, administered intra-abdominally, in a rat peritonitis model. Eur Surg Res 1996 Jul;28(4):287-94.
(19) Khurrum BM, Hua ZR, Batista O, Uriburu JP, Singh JJ, Weiss EG, et al. Percutaneous postoperative intra-abdominal abscess drainage after elective colorectal surgery. Tech Coloproctol 2002 Dec;6(3):159-64.
(20) Lamme B, Boermeester MA, Reitsma JB, Mahler CW, Obertop H, Gouma DJ. Meta-analysis of relaparotomy for secondary peritonitis. Br J Surg 2002 Dec;89(12):1516-24.
(21) Renckens R, Roelofs JJ, Florquin S, de Vos AF, Pater JM, Lijnen HR, et al. Endogenous tissue-type plasminogen activator is protective during Escherichia coli-induced abdominal sepsis in mice. J Immunol 2006 Jul 15;177(2):1189-96.
(22) Faist E, Schinkel C, Zimmer S. Update on the mechanisms of immune suppression of injury and immune modulation. World J Surg 1996 May;20(4):454-9.
(23) Damazo AS, Yona S, Flower RJ, Perretti M, Oliani SM. Spatial and temporal profiles for anti-inflammatory gene expression in leukocytes during a resolving model of peritonitis. J Immunol 2006 Apr 1;176(7):4410-8.
(24) Blot S, De Waele JJ. Critical issues in the clinical management of complicated intra-abdominal infections. Drugs 2005;65(12):1611-20.
(25) Rau B, Kruger CM, Schilling MK. Procalcitonin: improved biochemical severity stratification and postoperative monitoring in severe abdominal inflammation and sepsis. Langenbecks Arch Surg 2004 Apr;389(2):134-44.
(26) Sutton CD, Marshall LJ, Williams N, Berry DP, Thomas WM, Kelly MJ. Colo-rectal anastomotic leakage often masquerades as a cardiac complication. Colorectal Dis 2004 Jan;6(1):21-2.
(27) Bell WR. Present-day thrombolytic therapy: therapeutic agents--pharmacokinetics and pharmacodynamics. Rev Cardiovasc Med 2002;3 Suppl 2:S34-S44.
(28) Deitcher SR JMR. Pharmacologic and clinical characteristics of thrombolytic agents. Rev Cardiovasc Med 2003 Mar;2(Suppl.):S25-S33.



Chapter 6

Plasminogen activator, but not systemic antibiotic therapy, prevents abscess formation in secondary peritonitis in the rat

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ABSTRACT

Background
Intra-abdominal abscesses are sources for recurrent or ongoing abdominal sepsis. They constitute an important target for prevention or treatment after surgery for peritonitis. Prior experimental data suggest intraperitoneal fibrinolytic therapy to be effective and feasible, but this was only established in the absence of antibiotics

Method
Peritonitis was induced via intra-abdominal injection of a faces/bacteria mixture in rats. Surgical debridement was performed after 1 h. Animals were treated with antibiotics (ceftriaxone plus metronidazole for 3 days), recombinant tissue plasminogen activator (rtPA for 1 day) or both. Abdominal fluid samples were taken at 24, 72 and 120h for cytokine measurements and cell counts. After 5 days the abdomen was inspected for the presence of abscesses.

Results
Antibiotics did not significantly affect abscess formation (median number 4.5 in controls). RtPA significantly reduced the number of rats with abscesses and the abscess load per rat, both in absence and presence of antibiotic therapy (median number 0 in both rtPA groups). No adverse side effects were observed and no meaningful differences in the local inflammatory response were found.

Conclusion
rtPA consistently reduces abscess formation in a model for the surgical treatment of secondary peritonitis in the rat: it thus represents a promising adjuvant to conventional therapy.

INTRODUCTION

Sepsis and the systemic inflammatory response syndrome are the most common causes of death in adult intensive care units(1;2). Intra-abdominal infection is a well-known cause of severe sepsis and remains associated with a significant morbidity and mortality(3). In secondary peritonitis the gastrointestinal barrier is disrupted which leads to intra-abdominal infection caused by microbial pathogens or their products(4). Treatment is based on three modalities: surgery, antibiotic treatment and restoration and preservation of organ perfusion and oxygenation. Although improvements have been made over the years in all three, mortality and morbidity rates have hardly improved(5). Despite adequate treatment residual or recurrent infection is relatively frequent, necessitating a relaparotomy in 42% of all cases(6).

Intra-abdominal abscesses are well-known sources for recurrent or ongoing abdominal sepsis and therefore constitute an important target for prevention or elimination(7). During peritonitis both intra-abdominal coagulation and fibrinolysis are up-regulated to a different degree, resulting in a net increase of coagulation activity(8-10). As a consequence, fibrin deposits will form that serve to sequestrate the source of infection but also constitute a nidus of intra-abdominal abscesses.

In order to prevent abscess formation, attempts were made to enhance fibrinolytic activity with recombinant tissue-type plasminogen activator (rtPA) in rats with generalized peritonitis(11;12). While a significant reduction of abscess formation was observed, this was only achieved at the cost of increased mortality. The latter could be largely prevented by a single high dose of potentially nephrotoxic gentamycin(13). More recently, 'proof of principle' was supplied that such a beneficial effect of rtPA, if administered differently, is possible without adverse side effects(14). These experiments were performed in the absence of systemic antibiotics. Clinically the essential role of antibiotic therapy as an adjunct to surgical treatment of peritonitis is firmly established(15). Therefore in the current study, the effect of clinically relevant systemic antibiotics, delivered in the first days after surgical therapy of induced peritonitis, on intra-abdominal abscess formation have been investigated in the rat. These experiments were conducted both in the absence and presence of intraperitoneally applied rtPA.

MATERIALS AND METHODS

Experimental design

Seventy-two animals were randomly divided into four equal groups. Peritonitis was induced by intraperitoneal injection of 2 ml of a fecal suspension containing 10^4 cfu B. fragilis (strain 25285 from the American Type Culture Collection) and 10^5 cfu E. coli (strain 25922 from the American Type Culture Collection), respectively(16). One hour after inoculation, the rats were anesthetized by inhalation of a mixture of isoflurane-oxygen-nitrous oxide. The abdominal cavity was debrided including partial omentectomy and rinsed with 5 ml of sterile saline of 38°C. The abdomen was closed with a running suture of 4.0 Polyglactin 910 (Vicryl®, Ethicon, Amersfoort, The Netherlands), the skin was closed with staples. Rats received 5 ml normal saline subcutaneously for resuscitation.

The first (control) group received no further drug treatment, the second group (rtPA) received rtPA intra-abdominally, the third group systemic antibiotics (AB) and the fourth group antibiotics plus rtPA (rtPA+AB group). Human rtPA (1.25 mg in 2.5 ml saline, Actilyse®, Boehringer Ingelheim, Germany) was given as an intraperitoneal bolus, once at the end of surgery and then, percutanously, at 6 and 24 hours after inducing peritonitis. The groups that did not receive rtPA were treated with saline at the same time points.

Antibiotic therapy was given for the first three days and consisted of 15 mg/kg Metronidazole (Aventis Pharma B.V. Hoevelaken, The Netherlands), twice daily subcutaneously, and 15 mg/kg Ceftriaxone (Roche Nederland B.V. Mijdrecht, The Netherlands), twice daily intramuscularly. After five days the surviving rats were killed, a laparotomy was performed and the abdomen was inspected. Particular attention was paid to number and location of abscesses as well as to signs of intra-abdominal bleeding. The location of abscesses was divided into diaphragmatic, right and left paracolic area. All fibrin-encased purulent collections were considered to represent abscesses. All abscesses were scored semi quantitatively according to their diameter: < 1 mm: 1; 1 - 2 mm: 2 and > 2 mm: 3. For each rat, the total number represented the abscess score. Generalized peritonitis was defined as the presence of either diffuse purulent abdominal fluid, diffuse redness and oedema of the peritoneum or diffuse fibrinous adhesion between intra-abdominal organs.

Animals

Male Wistar rats weighing 250-280 g (Harlan BV, Horst, the Netherlands) were housed two per cage and accustomed to laboratory conditions for five days before the start of the experiment. All animals were weighed daily and had free access to water and standard rodent chow throughout the entire experimental period (Hope Farms, Woerden, the Netherlands). The Animal Ethics Review Committee of the Radboud University Nijmegen approved the study

Sample collection

At 24 h after inducing peritonitis, 500 microliter blood samples were taken for culture by orbital plexus puncture under general anaesthesia. Samples were collected in sterile tubes containing 50 IU of heparin.

Peritoneal fluid samples were collected under general anaesthesia at 24, 72 and 120 h after bacterial inoculation. After reopening the abdomen, 5 ml of sterile saline of 38°C was installed in the abdominal cavity. The abdomen was gently massaged and at least 2 ml of fluid were withdrawn. The samples were centrifuged for 10 min at 750 x g and supernatants stored at -80°C until further analysis.

Sample analysis

Heparinised blood samples (300 µl) were cultured for the presence of E. coli. Serial dilutions in Brain Heart Infusion broth were plated onto MacConkey blood agar plates. After 48 h of incubation at 37°C bacteria were identified and counted.

Cells were counted in the samples of peritoneal fluid after dilution of the sediment with saline to a concentration of 50.000 cells/ml and staining with May-Grunwald Giemsa reagent. Levels of tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-10 (IL-10) were measured in peritoneal fluid at 24 and 72 h. All assays were performed using commercially available ELISA kits (Endogen®, Pierce Biotechnology Inc, Rockford IL, USA). According to the manufacturer, the sensitivities for the various assays were < 10 pg/ml for TNF-α, < 16 pg/ml for IL-6 and <3 pg/ml for IL-10, respectively.

Statistics

Statistical analysis was performed using two-way Kruskal-Wallis test followed by Dunn's multiple comparison test and Mann Whitney test. Trend analysis for the abscess related parameters was performed using a Pearson chi square test and COX regression. P values < 0.05 were considered significant.

RESULTS

Clinical course and survival

All rats became ill after inoculation, as characterized by lack of movement, pilo-erection and anorexia. Body weight decreased consistently and significantly ($p<0.05$) and stabilized at the 4th day. At this time, average weight loss in the control group was 9.3 %. The changes in body weight during the experimental period were similar in all groups. One rat in the rtPA group died at day 2. Generalized peritonitis was apparent on post mortem examination. No bleeding complications were observed.

Abscesses

Intra-abdominal abscesses were found in 15 out of 18 rats in the control group and in 17 out of 18 in the group treated with antibiotics alone. Systemic antibiotics alone did not reduce the number of rats with abscesses, while rtPA did, also in the presence of antibiotics (Figure 1). Similar results were obtained for the number of abscesses per rat (Figure 2A) and the abscess score (Figure 2B), which showed the same pattern and significant differences between groups.

Overall, abscesses were found at preferential locations, i.e. in the sub diaphragmatic area and in the paracolic gutters. In particular, significantly more rats with abscesses at the diaphragm and the left paracolic gutter were observed in controls as well as in the AB group as compared to the rtPA and rtPA+AB groups. In addition, significantly more rats with abscesses at the right paracolic gutter were observed in the controls as compared to the rtPA group (Fisher exact test $p<0.01$, table 1). When comparing total numbers of abscesses at the preferential locations similar results were found.

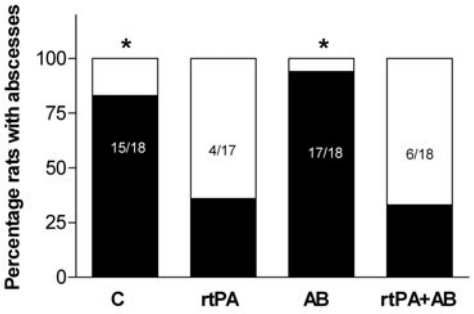

Figure 1. **Percentage of rats with abscesses.**
The numbers within the columns indicate the actual number of rats with abscesses (represented as black bars) versus the total number of survivors in that group. *indicates a significant ($p<0.001$, chi-square) difference with the rtPA-treated groups.

	n	Diaphragm	Right paracolic	Left paracolic
Controls	18	12*	6**	15*
rtPA	17	0	0	4
Antibiotics	18	8*	9**	12*
rtPA + antibiotics	18	0	3	3

Table 1a. **Distribution of abscesses.** Number of rats with abscesses at preferential locations.
*significantly (p<0.05) more abscesses as compared to rtPA and rtPA + antibiotics. **significantly (p<0.05) more abscesses as compared to rtPA.

	n	Diaphragm	Right paracolic	Left paracolic
Controls	18	22*	7**	44*
rtPA	17	0	0	5
Antibiotics	18	13*	12**	28*
rtPA + antibiotics	18	0	3	6

Table 1b. **Distribution of abscesses.** Total number of abscesses at preferential locations.
*significantly (p<0.05) more abscesses as compared to rtPA and rtPA + antibiotics. **significantly (p<0.05) more abscesses as compared to rtPA.

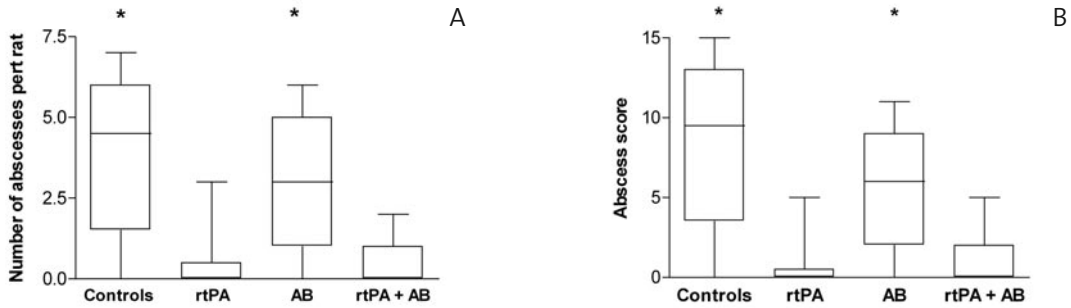

Figure 2a en 2b. **Abscess data.** Both the number of abscesses per rat (A) and the abscess score (B) are depicted. Data are represented as medians (measurements in all surviving animals) with 25-75 percentiles in boxes and 5-95 percentiles represented as vertical lines. *indicates a significant (p<0.001, Kruskal-Wallis plus Dunn's test) difference with the rtPA-treated groups.

Blood cultures

Positive blood cultures were found in 11-44% of all rats. The median number (range) of colony forming units per rat varied from 20 (20-100) cfu/ml to 220 (20-1200) cfu/ml. There were no significant differences between the groups with respect to either the number of rats with positive cultures or the mean number of colony forming units per group.

Cytokines

The levels of IL-6 and IL-10 significantly declined in all groups from 24 h to 72 h, while levels of TNF-α significantly increased over this period (Figure 3).

IL-6 levels at 24 h were significantly higher in the rtPA group than in the rtPA+AB group ($p < 0.01$). At 72 h IL-6 levels did not significantly differ between groups. Levels of TNF-α and IL-10 did not significantly differ between groups at either 24 or at 72 h.

Cells

At all time points the total cell numbers in peritoneal samples did not significantly differ between groups. In the controls, the percentage of neutrophils declined with time (from 77% at 24 h to 19% at 120 h). Eosinophils and macrophages increased, from 6 to 42% and from 17 to 32%, respectively. Treatment with antibiotics did not affect these numbers. In general, treatment with rtPA did result in lower percentages of neutrophils and higher percentages of eosinophils, although differences were relatively small and only significant by exception.

DISCUSSION

Intra-abdominal abscess formation after surgical treatment of generalized peritonitis in rats can be reduced by intraperitoneal application of rtPA, but not by antibiotics. Neither rtPA nor antibiotics, administered either alone or in combination, influence mortality or alter the inflammatory response in the abdominal cavity.

The present study is one out of a recent series of sequential experiments in rats with generalized peritonitis. Until now, systemic antibiotics were deliberately not applied in order to study potential effects of interventions step by step. Intraperitoneal applied fibrinolytic therapy with rtPA or urokinase was shown to be effective in preventing intra-abdominal abscess formation in rats with generalized

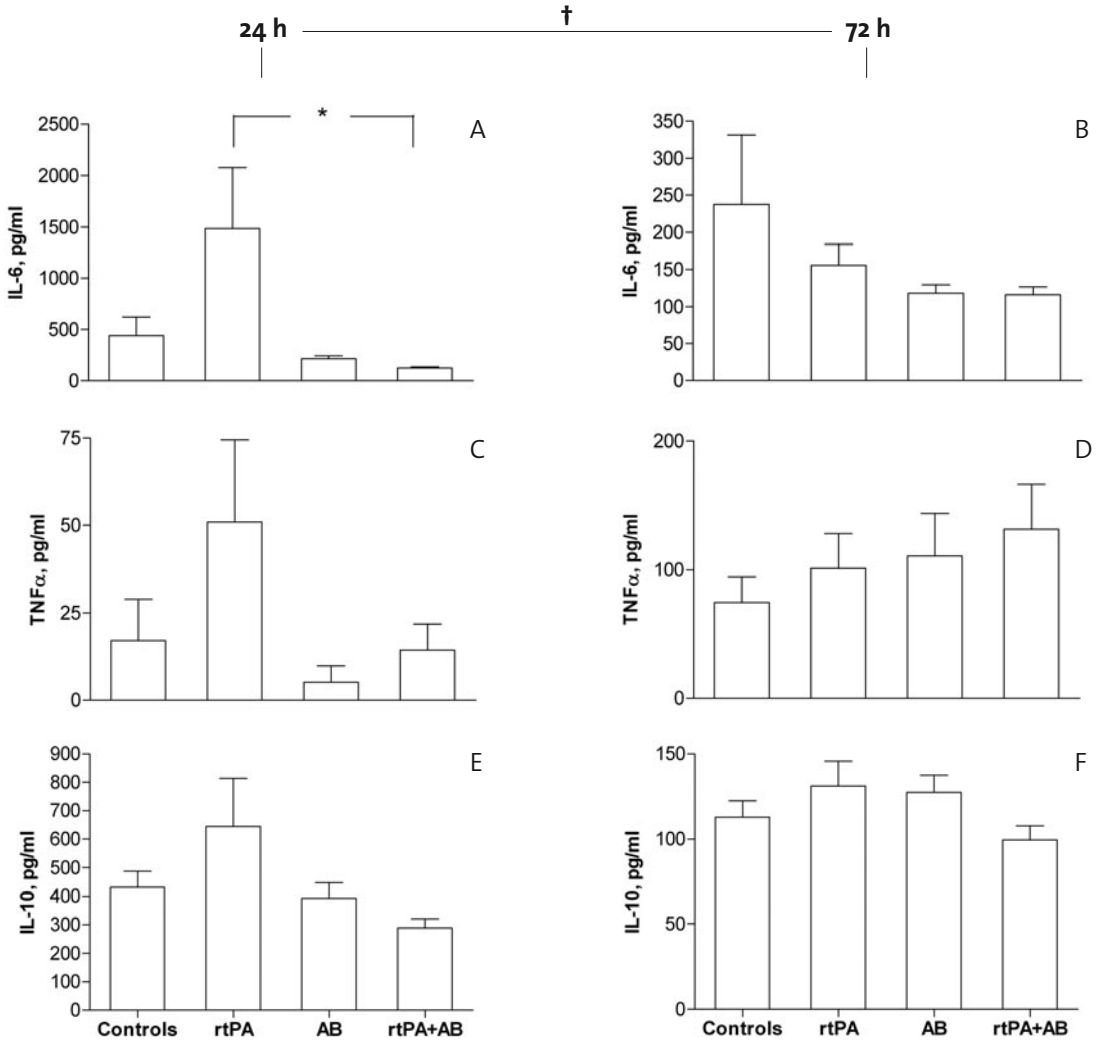

Figure 3. **Cytokines in abdominal fluid.** Data for IL-6 (A,B), TNF-α (C,D) and IL-10 (E,F) are depicted as means (measurements in all surviving animals) plus SEM represented as vertical lines. * indicates a significant ($p<0.01$, Kruskal-Wallis plus Dunn's test) difference between groups. † indicates a significant difference for cytokine levels at 24 versus 72 h.

peritonitis(14;17). Three doses of rtPA, given over 24 hours, were more effective than either a single or two consecutive doses in preventing abscess formation(14). RtPA is the main activator of plasmin, which in turn is the key enzyme in fibrin degradation(18) and can thus act to break down abscess walls that are mainly composed of fibrin(19). The latter is formed as a result of the physiologic reaction to any kind of trauma to the peritoneum, e.g. peritonitis. After peritoneal trauma, a fibrin matrix develops which acts as a scaffold for repair cells to migrate and exert their functions. The fibrinolytic system removes the fibrin in order to establish physiologic, adhesion-free healing(20).

The present experiment confirms recent studies on the effects of rtPA alone. The current rtPA dose is based on its clinical use for treatment of thrombo-embolic disorders(21), considering that the coagulation activity rate in the rat is elevated by a factor 2.7 compared to man(22). Abscess reduction by application of rtPA was reflected not only in the percentage of rats without abscesses or the median number of abscesses per rat. The distribution of abscesses changed as well. In the rtPA treated groups significantly fewer abscesses were found in the paracolic gutter and the sub-diaphragmatic spaces. This is explained by the cephalad peritoneal fluid stream transporting rtPA from the abdominal cavity via the paracolic gutters to the sub-diaphragmatic spaces(23).

Improvements of outcome of peritonitis have so far been achieved by subduing the systemic effects of abdominal sepsis. Timely delivery of adequate antibiotic therapy is of paramount importance for survival in the clinical setting(15). Data on strategies to improve control of the local infection and inflammation remain equivocal(6;15;24-26).The combination of metronidazole and ceftriaxone is known to be effective in the treatment of secondary peritonitis in humans(27;28). In animal models this antibiotic regimen induced an improvement of survival(29;30). In the current study mortality was only 1.3%, precluding any conclusions with respect to the possible effect of systemic antibiotic therapy on survival. The current study shows that systemic antibiotic therapy has no effect on abscess formation. This may have two causes. First, fibrin renders bacteria unreachable for antibiotics and the immune system in the abdominal cavity. Second, antibiotics may be less effective due to the low pH, protein binding and degradation by bacterial enzymes in abscesses(31).

Inflammatory cytokines and cell counts were studied in addition to abscess formation in order to detect a possible effect explaining the action mechanism of rtPA whether or not in combination with antibiotics. RtPA may have an immune modulatory effect in the abdominal cavity, although the precise mechanism is not understood(32;33). The available data on the influence of rtPA on the local inflammatory response in peritonitis are mainly experimental and open to multiple interpretations.

Endogenous tPA deletion or targeting in mice leads to impairment of the inflammatory response to E. coli peritonitis. In tPA-/- mice similar peritoneal neutrophil counts but higher local bacterial loads and higher levels of neutrophil attracting chemokines were found, if compared to wild type mice(32). These results suggest a reduced migratory response. Intra-abdominal application of exogenous tPA in a caecal ligation and puncture model reduced bacterial load and leukocyte influx. However, the effects on cytokine response were equivocal(34). Cook et al. showed that the number of macrophages in peritoneal exudate was reduced in experimental peritonitis, but could be increased by applying exogenous tPa or plasmin(35). Altogether, at this point, the implications for the clinical setting from the above mentioned results remain unclear.

In the current study equivocal observations regarding alteration of the local inflammatory response were made. At 24 h a significantly higher level of pro inflammatory IL-6 was measured in the rtPA treated group as compared to the rtAP+AB group. Apparently, the addition of antibiotics to rtPA reduced the pro-inflammatory effect of rtPA, although there was no difference between the rtPA group and the controls. A similar finding was observed with respect to TNF-α levels at 24 h. No effect on levels of anti-inflammatory IL-10 was observed. In the existing literature there are no data on the possible interaction of rtPA and antibiotics, as used in the present study, with respect to inflammatory response. Regarding the cellular response, significant differences were observed only between controls and the rtPA group. Yet, these findings were ambiguous because they involved a different cell type at each time point. The addition of antibiotics to rtPA treatment did not affect the cellular response.

It is concluded that intra-abdominal application of rtPA consistently reduces abscess formation and therefore is a promising adjuvant to systemic antibiotics in the therapy of secondary peritonitis. It should be noted that for clinical practice the necessity for repeat dosing might require the standard use of an indwelling device. Antibiotic treatment alone does not contribute significantly to reduction of abscess formation but is important in reducing the systemic effects of peritonitis. These results further corroborate the potential role of fibrinolytic therapy in optimal treatment of secondary peritonitis and support advancement into a phase two clinical trial.

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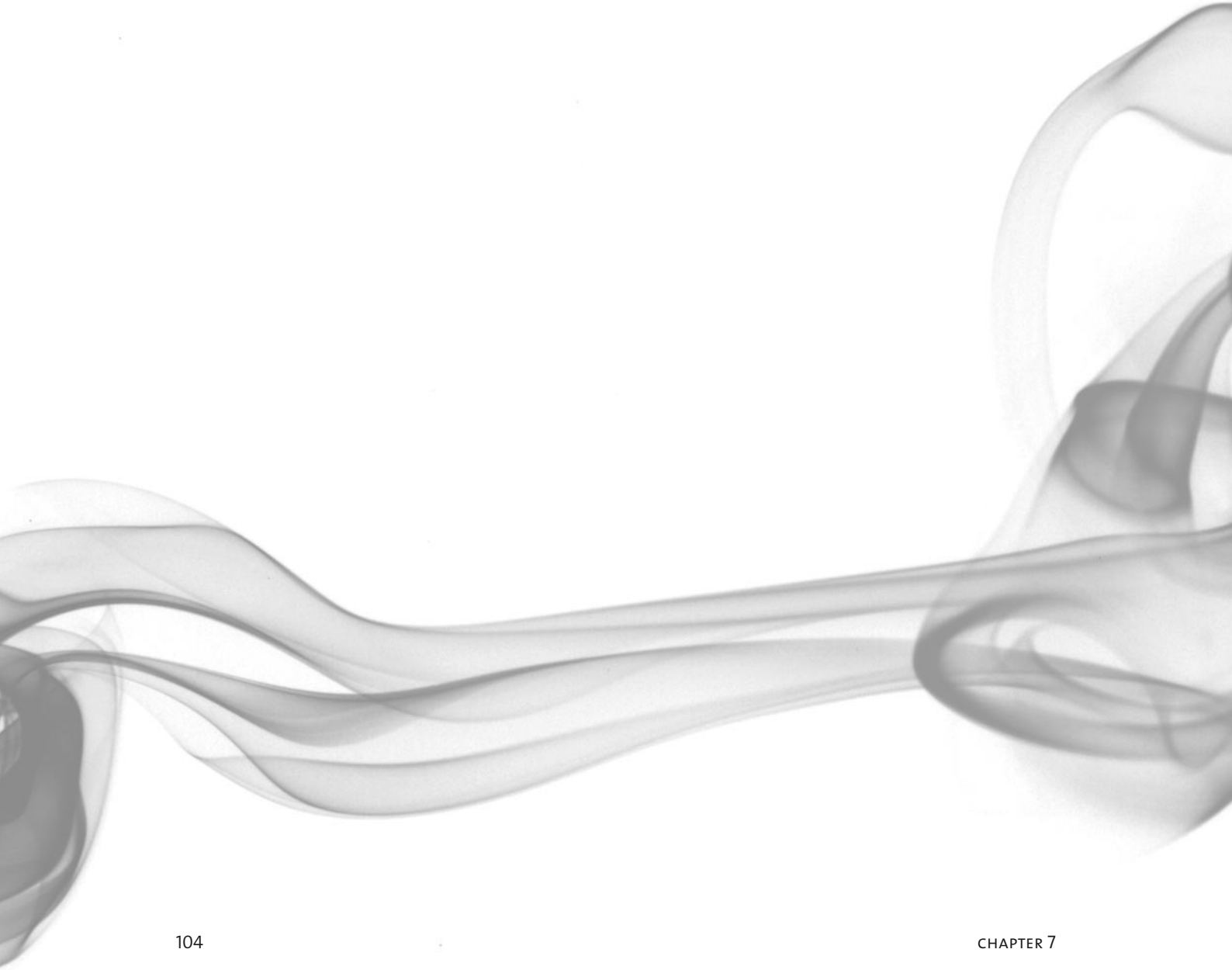
REFERENCE LIST

(1) Sihler KC, Nathens AB. Management of severe sepsis in the surgical patient. Surg Clin North Am 2006 Dec;86(6):1457-81.
(2) Gullo A, Bianco N, Berlot G. Management of severe sepsis and septic shock: challenges and recommendations. Crit Care Clin 2006 Jul;22(3):489-501, ix.
(3) Lamme B, Boermeester MA, Reitsma JB, Mahler CW, Obertop H, Gouma DJ. Meta-analysis of relaparotomy for secondary peritonitis. Br J Surg 2002 Dec;89(12):1516-24.
(4) Wittmann DH, Schein M, Condon RE. Management of secondary peritonitis. Ann Surg 1996 Jul;224(1):10-8.
(5) Emmanuel K, Weighardt H, Bartels H, Siewert JR, Holzmann B. Current and future concepts of abdominal sepsis. World J Surg 2005 Jan;29(1):3-9.
(6) van Ruler O., Mahler CW, Boer KR, Reuland EA, Gooszen HG, Opmeer BC, et al. Comparison of on-demand vs planned relaparotomy strategy in patients with severe peritonitis: a randomized trial. JAMA 2007 Aug 22;298(8):865-72.
(7) Marshall JC, Innes M. Intensive care unit management of intra-abdominal infection. Crit Care Med 2003 Aug;31(8):2228-37.
(8) Holmdahl L. The role of fibrinolysis in adhesion formation. Eur J Surg Suppl 1997;(577):24-31.
(9) Holmdahl L, Falkenberg M, Ivarsson ML, Risberg B. Plasminogen activators and inhibitors in peritoneal tissue. APMIS 1997 Jan;105(1):25-30.
(10) van Veen SQ, Levi M, van Vliet AK, Florquin S, van Gulik TM, Boermeester MA. Peritoneal lavage with activated protein C alters compartmentalized coagulation and fibrinolysis and improves survival in polymicrobial peritonitis. Crit Care Med 2006 Nov;34(11):2799-805.
(11) Rotstein OD, Kao J. Prevention of intra-abdominal abscesses by fibrinolysis using recombinant tissue plasminogen activator. J Infect Dis 1988 Oct;158(4):766-72.
(12) van Goor H, de Graaf JS, Kooi K, Sluiter WJ, Bom VJ, van der MJ, et al. Effect of recombinant tissue plasminogen activator on intra-abdominal abscess formation in rats with generalized peritonitis. J Am Coll Surg 1994 Oct;179 (4):407-11.
(13) van Goor H, de Graaf JS, Kooi K, Bleichrodt RP. Gentamycin reduces bacteremia and mortality rates associated with the treatment of experimental peritonitis with recombinant tissue plasminogen activator. J Am Coll Surg 1995 Jul;181(1):38-42.
(14) Buyne OR, Bleichrodt RP, van Goor H., Verweij PE, Hendriks T. Tissue-type plasminogen activator prevents formation of intra-abdominal abscesses after surgical treatment of secondary peritonitis in a rat model. Int J Colorectal Dis 2007 Jul;22(7):819-25.
(15) Boermeester MA. Surgical approaches to peritonitis. Br J Surg 2007 Nov;94(11):1317-8.
(16) Buyne OR, Bleichrodt RP, Verweij PE, Groenewoud HM, van Goor H., Hendriks T. A peritonitis model with low mortality and persisting intra-abdominal abscesses. Int J Exp Pathol 2006 Oct;87(5):361-8.

(17) Buyne OR, Bleichrodt RP, van Goor H, Verweij PE, Hendriks T. Both Tissue-type Plasminogen Activator And Urokinase Prevent Intra-abdominal Abscess Formation After Surgical Treatment Of Peritonitis In The Rat. Surgery 2008.
(18) Collen D, Lijnen HR. Thrombolytic agents. Thromb Haemost 2005 Apr;93(4):627-30.
(19) Reijnen MM, Bleichrodt RP, van Goor H. Pathophysiology of intra-abdominal adhesion and abscess formation, and the effect of hyaluronan. Br J Surg 2003 May;90(5):533-41.
(20) Tarhan OR, Barut I, Sezik M. An evaluation of normal saline and taurolidine on intra-abdominal adhesion formation and peritoneal fibrinolysis. J Surg Res 2008 Jan;144(1):151-7.
(21) Deitcher SR JMR. Pharmacologic and clinical characteristics of thrombolytic agents. Rev Cardiovasc Med 2003 Mar;2(Suppl.):S25-S33.
(22) van Goor H. In vitro studies on the effect of recombinant tissue plasminogen activator on the rat fibrinolytic system. Thesis: Fibrinolytic therapy in generalized peritonitis to prevent intraabdominal abscess formation. Nijmegen: 1996. p. 125-34.
(23) Raptopoulos V, Gourtsoyiannis N. Peritoneal carcinomatosis. Eur Radiol 2001;11(11):2195-206.
(24) Schein M. Surgical management of intra-abdominal infection: is there any evidence? Langenbecks Arch Surg 2002 Apr;387(1):1-7.
(25) Tomita H, Osada S, Miya K, Matsuo M. Delayed recurrence of postoperative intra-abdominal abscess: an unusual case and review of the literature. Surg Infect (Larchmt) 2006 Dec;7(6):551-4.
(26) van Till JW, Lamme B, van Esch TJ, van der PT, van Gulik TM, Boermeester MA. Surgical therapy attenuates abdominal and extra-abdominal inflammation in experimental peritonitis. Eur Surg Res 2006;38(2):76-82.
(27) Wong PF, Gilliam AD, Kumar S, Shenfine J, O'Dair GN, Leaper DJ. Antibiotic regimens for secondary peritonitis of gastrointestinal origin in adults. Cochrane Database Syst Rev 2005;(2):CD004539.
(28) Orozco H, Arch J, Medina-Franco H, Pantoja JP, Gonzalez QH, Vilatoba M, et al. Molgramostim (GM-CSF) associated with antibiotic treatment in nontraumatic abdominal sepsis: a randomized, double-blind, placebo-controlled clinical trial. Arch Surg 2006 Feb;141(2):150-3.
(29) Turnbull IR, Wlzorek JJ, Osborne D, Hotchkiss RS, Coopersmith CM, Buchman TG. Effects of age on mortality and antibiotic efficacy in cecal ligation and puncture. Shock 2003 Apr;19(4):310-3.
(30) Bauhofer A, Torossian A, Lorenz W, Middeke M, Plaul U, Schutz P, et al. Dependence of positive effects of granulocyte colony-stimulating factor on the antibiotic regimen: evaluation in rats with polymicrobial peritonitis. World J Surg 2004 Aug;28(8):834-44.
(31) Wagner C, Sauermann R, Joukhadar C. Principles of antibiotic penetration into abscess fluid. Pharmacology 2006;78(1):1-10.
(32) Renckens R, Roelofs JJ, Florquin S, de Vos AF, Pater JM, Lijnen HR, et al. Endogenous tissue-type plasminogen activator is protective during Escherichia coli-induced abdominal sepsis in mice. J Immunol 2006 Jul 15;177(2):1189-96.
(33) Roelofs JJ, Rouschop KM, Teske GJ, Claessen N, Weening JJ, van der PT, et al. The urokinase plasminogen activator receptor is crucially involved in host defense during acute pyelonephritis. Kidney Int 2006 Dec;70(11):1942-7.

(34) van Veen SQ, Meijers JC, Levi M, van Gulik TM, Boermeester MA. Effects of intra-abdominal administration of recombinant tissue plasminogen activator on coagulation, fibrinolysis and inflammatory responses in experimental polymicrobial peritonitis. Shock 2007 May;27(5):534-41.
(35) Cook AD, Vlahos R, Massa CM, Braine EL, Lenzo JC, Turner AL, et al. The effect of tissue type-plasminogen activator deletion and associated fibrin(ogen) deposition on macrophage localization in peritoneal inflammation. Thromb Haemost 2006 Apr;95(4):659-67.



Chapter 7

Tissue-type plasminogen activator prevents abscess formation but does not affect healing of bowel anastomoses and laparotomy wounds in rats with secondary peritonitis

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ABSTRACT

Background
Intra-abdominal application of recombinant tissue-type plasminogen activator (rtPA) can decrease the rate of abscess formation in a rat peritonitis model. Before using rtPA clinically, its effects on healing of bowel anastomoses and laparotomy wounds should be investigated.

Methods
Peritonitis was induced in 148 male Wistar rats via intra-abdominal injection of a feces/bacteria mixture. Laparotomy, operative debridement and construction of a colo-colostomy after a limited colectomy or ileo-ileostomy after a limited ileal resection were performed after 1 h. All animals received antibiotics (ceftriaxone plus metronidazole). In addition to untreated controls, other animals received rtPA in either of three dosing schemes, starting immediately after operation or 24 h afterwards. Wound strength and hydroxyproline content of the wound were analyzed after 3 or 7 days.

Results
Mortality was 2% and manifestations of excessive bleeding were virtually absent. RtPA significantly decreased the rate of abscess formation. Neither bursting pressure nor breaking strength of the anastomoses was affected by any of the rtPA protocols. The same was true for wound strength in the abdominal fascia. Also, wound hydroxyproline content and wound architecture remained unchanged after rtPA administration.

Conclusion
Intraperitoneal rtPA administration consistently and significantly decreased the rate of abscess formation but did not affect wound healing. Clinical studies investigating its potential as an adjunct in the treatment of secondary peritonitis may be warranted.

INTRODUCTION

Secondary peritonitis is a life-threatening disease that is caused usually by perforation of the digestive tract(1). The therapy of secondary peritonitis is based on surgical source control, resuscitation, and systemic antibiotic therapy(2;3). Surgical therapy often involves laparotomy and bowel resection. Constructing an intestinal anastomosis is justified in selected patients(4;5). Despite optimal treatment, intra-abdominal abscesses occur in 15-25% of patients and can lead to ongoing and recurrent abdominal sepsis(6;7).

During peritonitis both intra-abdominal coagulation and fibrinolysis are up-regulated to a different degree, resulting in a net increase of coagulation activity and deposition of fibrin. These fibrin deposits contain viable bacteria that become unreachable for the local immune defenses in the abdominal cavity and for antibiotics and thus form the nidus for intra-abdominal abscesses(8;9). Fibrinolytic therapy may offer an attractive way to reduce the risk of abscess formation.

Recently, we reported new preclinical evidence that treatment with recombinant tissue-type plasminogen activator (rtPA) after operative debridement for secondary peritonitis may prevent abscess formation without unwanted side-effects(10;11). Before initiating a clinical study, it becomes necessary to ascertain that rtPA treatment does not interfere with the healing of abdominal wall fascia or bowel anastomoses, which would increase the risk of burst abdomen or anastomotic dehiscence.

Fibrin deposition is intrinsic to early wound repair and provides a provisional scaffold for ingrowth of fibrocollagenous tissue and for cell migration. An excessive presence of fibrinolytic factors such as rtPA may induce premature degradation of this provisional matrix. In addition, the plasminogen activator/plasmin system can activate the matrix metalloproteinases, which are already induced during wound repair(12). Both processes could thus result in unwanted matrix degradation, decrease in the suture-holding capacity, and ultimately result in decreased wound strength. There are few data on the effects of exogenous rtPA on wound healing, although a detrimental effect has been suggested(13).

We designed a comprehensive experiment to study the effect of the intraperitoneal application of rtPA on healing of bowel anastomoses and abdominal wall fascia after operative debridement of secondary peritonitis in the rat. We have previously validated the therapeutic dosage scheme of rtPA in decreasing the rate of abscess formation(10;14).

MATERIALS AND METHODS

Design of the study

Male Wistar rats weighing 250-280 g (Harlan BV, Horst, the Netherlands) were housed two per cage and accustomed to laboratory conditions for five days before the start of the experiment. Peritonitis was induced in all rats, and 1 h later, operative debridement was performed. Therafter, animals underwent resection and anastomosis in either colon or ileum. Intraperitoneal rtPA treatment consisted of 3 doses administered over a 24 h period. The first dose was given either immediately after operation or 1 day later. Animals were killed 3 or 7 days after operation. All animals were weighed daily and had free access to water and standard rodent chow throughout the entire experimental period (Hope Farms, Woerden, the Netherlands). The Animal Ethics Review Committee of the Radboud University Nijmegen approved the study.

Induction and treatment of peritonitis

Peritonitis was induced in all rats by a transcutaneous, intraperitoneal injection of 2 ml of a fecal suspension containing 10^4 colony forming units (cfu) of Bacteroides fragilis (strain 25285 from the American Type Culture Collection) and 10^5 cfu of Escherichia coli (strain 25922 from the American Type Culture Collection), respectively(15)., One h after inoculation, operative therapy was performed. The rats were anesthetized by inhalation of a mixture of isoflurane (Abbott Laboratories, Queensborough, UK) -oxygen-nitrous oxide. The abdominal cavity was debrided including partial resection of the omentum: all dead or macroscopically infected tissue as well as any contaminated material was removed and the abdominal cavity was irrigated with 5 ml of sterile 0.9% NaCl of 38°C. Antibiotic therapy was given for the first three days and consisted of 15 mg/kg metronidazol (Aventis Pharma B.V. Hoevelaken, The Netherlands), twice daily subcutaneously, and 15 mg/kg ceftriaxone (Roche Nederland B.V. Mijdrecht, The Netherlands), twice daily intramuscularly.

Study groups

In the first experiment, colonic resection and anastomosis were performed in 120 rats immediately after debridement of the abdominal cavity. The animals were randomized into four groups of 30 rats. In the first group (rtPA), human rtPA (Actilyse®, Boehringer Ingelheim, Germany), in a dose of 1.25 mg in 2.5 ml of 0.9% NaCl, was given as an intraperitoneal bolus, once at the end of the operation and

then, percutanously, at 6 and 24 h after bacterial inoculation. The second group (rtPA-low) was treated identically but each rtPA dose was 0.25 mg. In the third group (rtPA-del), three intraperitoneal doses of 1.25 mg rtPA were given at 24, 30 and 48 h after bacterial inoculation. The control group was similar to the rtPA group but received intraperitoneal 0.9% NaCl only. Within each group, half of the animals (15 in each group) were killed at 3 and 7 days after operation, respectively. Twelve animals were used for assay of wound strength and the remaining for histology.
In the second experiment, ileal resection and anastomosis was performed in 28 rats immediately after debridement of the abdominal cavity. The animals were randomized into two groups of 14 rats. The first group received 1.25 mg of rtPA intraperitoneally after operation and,6 and 24 h after bacterial inoculation. The second group received intraperitoneal 0.9% NaCl only at the same time points. All rats were killed at the third postoperative day and used for determination of wound strength.

Bowel anastomoses

Procedures were performed under semi sterile conditions using a Zeiss operation microscope (Carl Zeiss AG, Oberkochen, Germany). In experiment 1, a 2 cm resection of the descending colon, 3 cm proximal to the peritoneal reflection was performed. Colonic continuity was restored by constructing an end-to-end anastomosis with 8 single-layer, inverting, interrupted sutures (Ethilon 8-0, Ethicon, Germany). In experiment 2, a similar resection and end-to-end anastomosis was performed in the distal ileum, 15 cm proximal to the cecum. During operations, body temperature was kept at 38°C using a heating pad and a lamp. Animals were monitored by means of pulse oximetry and temperature measuring devices. Intestines were covered with gauze pads soaked with 0.9% NaCl to minimize desiccation. To prevent dehydration, 5 mL of 0.9% NaCl was administered subcutaneously during the operation. Perioperative analgesia was given with buprenorphine, 0.02 mg/kg subcutaneously, twice daily for 2 days. Animals were weighed daily. All operative procedures were performed by the same investigator.

Analysis of abscess formation and wound strength

Rats were killed by CO/CO_2 asphyxiation at either the third or the seventh postoperative day, and the abdomen was inspected. Particular attention was paid to the number of abscesses and to signs of intra-abdominal bleeding. All fibrin-encased purulent collections were considered to represent abscesses, which were scored semi-quantitatively according to their diameter: < 1 mm: 1 point; 1 - 2 mm: 2 points and > 2 mm: 3 points. For each rat, the sum of these points represented the abscess score.

Generalized peritonitis was defined as the presence of either diffuse purulent abdominal fluid, diffuse redness and edema of the peritoneum, or diffuse fibrinous adhesion between intra-abdominal organs. Adhesions were dissected carefully without manipulation of the anastomosis. Presence of adhesions or anastomotic dehiscence was recorded. Segments containing the anastomoses were resected. To measure bursting pressure, the segments were infused (2 mL/min) with 0.9% NaCl containing methylene blue. The maximum pressure (mm Hg) recorded immediately before sudden loss of pressure was taken as the bursting pressure. The site of rupture (within or outside the anastomotic line) was noted. Subsequently, the segments were placed in a tensiometer, and the breaking strength (grams) was measured(16;17). To determine the abdominal wall strength, two full-thickness strips of 3 to 7 mm width and 20 mm length were cut from the abdominal wall between sutures. Breaking strength in these samples, containing fascia and muscle layer, was measured longitudinally in a tensiometer and expressed as gram/mm.

Biochemical analysis and histology

After measuring wound strength, the tissue samples were dissected from adhering tissue and debris, and 5 mm samples containing the anastomosis or the abdominal suture line of the midline fascia were frozen in liquid nitrogen and stored at -80°C until further processing. After lyophilization, tissue samples were weighed, pulverized, and lyophilized again. The hydroxyproline content, as a measure of the collagen content, was measured by high-performance liquid chromatography after hydrolysis with 6 N HCL acid and derivatization with dabsyl-chloride(18).

Sections of anastomoses and abdominal wall originating from separate animals that had not been subjected to strength measurements were washed in 0.9% NaCl, spread out, and fixed immediately in a 4% phosphate-buffered formaldehyde solution. Subsequently, the samples were dehydrated and embedded in paraffin blocks. Sections of 4 µm in thickness were stained with hematoxylin and eosin (H&E).

Statistics

The differences in rate of abscess formation between the control group and each of the experimental groups were tested using a Mann-Whitney test and employing a Bonferroni correction. Differences in wound strength and hydroxyproline content were analysed with a Kruskal-Wallis test with a Dunn correction for multiple comparison. All tests were two-sided; the level of significance was set at $p < 0.05$.

RESULTS

Experiment 1: colonic anastomoses

Clinical course and abscess formation: All rats became severely ill after inoculation, as characterized by lack of movement, pilo-erection, and anorexia. Body weight decreased during the first 4-5 days after operation and stabilized thereafter. The changes in body weight during the experimental period were similar in all groups (Figure 1). Overall survival rate was 117/120 (98%). No significant differences in survival between groups were observed. In one rat of the rtPA group that died prematurely, a subcutaneous hematoma and a small amount of intra-abdominal blood was found. No abnormalities were found in the two other rats that died prematurely, one from the rtPA group and one from the rtPA-del group. No signs of bleeding were observed in rats that survived until the end of the study. In all surviving rats, two or more signs of peritonitis were observed at post mortem examination.
Treatment with rtPA decreased the rate of abscess formation. At day 3, both the number of abscesses per rat and the abscess score were less in all the treatment groups when compared to the control group ($p<0.05$; figure 2). The number of rats without abscesses increased from 0/15 in the controls to 7/14, 5/15 and 1/15 in the rtPA, rtPA-low, and rtPA-del groups, respectively. On day 7, similar results were found (data not shown), with both abscess numbers and abscess score being less in the rtPA and rtPA-low groups than in the controls ($p<0.05$). No wound infections were observed.

Wound strength: In all groups, both anastomotic and fascial wound strength significantly increased from 3 to 7 days after operation ($p<0.05$). The increase in anastomotic breaking strength was similar in all groups (Figure 3A) and at neither time point was a difference between controls and rtPA-treated groups observed. The same was true for the anastomotic bursting pressure in the colon. At day 3, the bursting site was almost invariably within the suture line, while at day 7, bursting occurred usually outside the anastomosis in the adjacent tissue (Figure 4).
The breaking strength of the fascial wounds was very low after 3 days and increased rapidly thereafter (Figure 3B). Again, none of the rtPA treatment protocols resulted in measurable changes in wound strength.

Hydroxyproline content and histology: Neither hydroxyproline concentration nor hydroxyproline content at day 7 differed between groups, for either the colonic or fascial wounds (Table 1). While

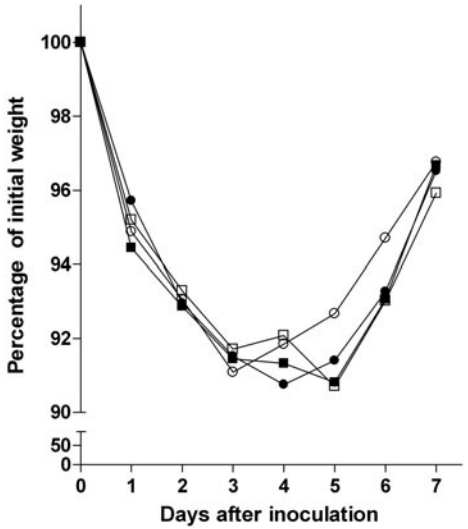

Figure 1. **Changes of body weight.**
Average weight is expressed as percentage of weight at the time of bacterial inoculation. ● Controls, ■ rtPA, □ rtPA-low, ○ rtPA-del

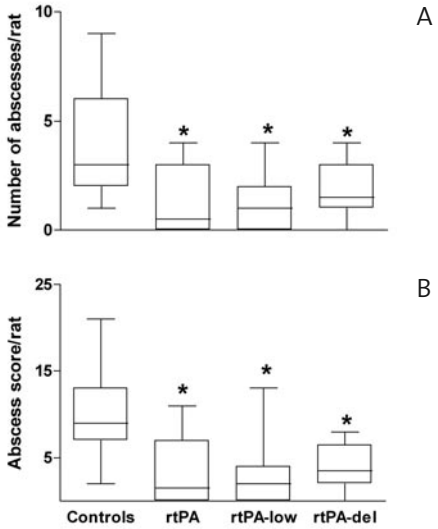

Figure 2. **Abscess formation.** Number of abscesses per rat (A) and the total abscess score per rat (B) after 3 days. Controls and rtPA-low: n = 15; rtPA and rtPA-del: n = 14. Data are depicted as medians with 5-95 percentiles in boxes and range represented as vertical lines. *: $p < 0.05$ vs untreated controls.

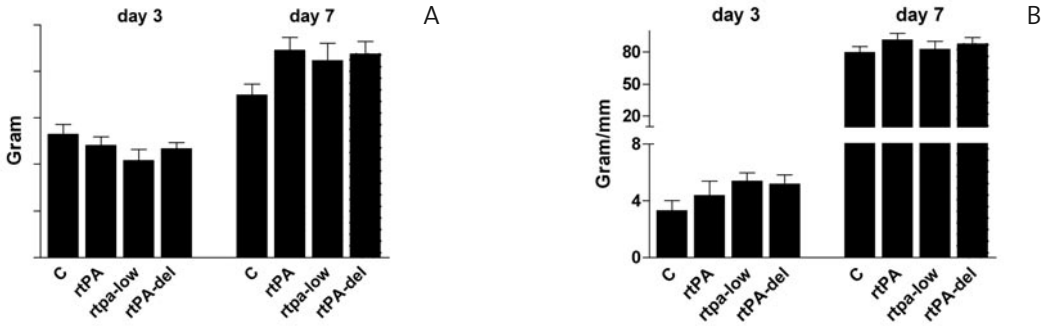

Figure 3a, 3b. **Wound breaking strength.**
Anastomotic (A) and fascial (B) breaking strength (B) at day 3 and day 7. Data are depicted as mean + standard error of mean (n=12).

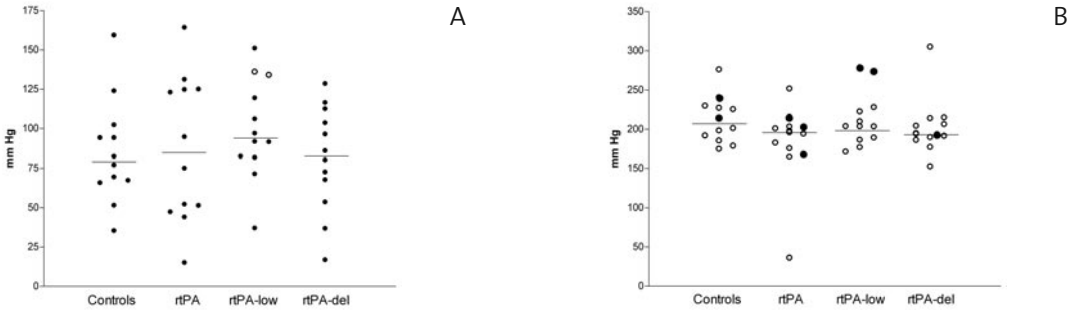

Figure 4. **Anastomotic bursting pressure.** Individual values for colonic anastomoses at day 3 (A) and day 7 (B). The vertical lines represent median values. ●bursting site within the anastomosis,○bursting outside the suture line.

comparison of the anastomotic hydroxyproline content indicated an overall difference between groups (p=0.033, Kruskal-Wallis), the (Dunn's) post test failed to demonstrate actual differences between the control group and any of the experimental groups.

A comprehensive examination of sections obtained from each group failed to reveal any obvious architectural differences between the groups at day 3 or at day 7. Semi-quantitative analysis also failed to indicate any differences between control and rtPA-treated groups with respect to histologic parameters such as epithelial damage, wound area surface, degree of necrosis, edema, mucosal repair, and cellular infiltration.

These findings are illustrated in figure 5, which gives typical examples of H/E sections of anastomoses in the colon from controls and animals from the rtPA group at day 3 and day 7 after operation.

	Hydroxyproline concentration		Hydroxyproline content	
	Colon	Fascia	Colon	Fascia
Controls	12.5 ± 2.1	24.9 ± 7	307 ± 56	95 ± 28
rtPA	11.4 ± 1.5	20.8 ± 5.7	251 ± 46	88 ± 17
rtPA-low	12.4 ± 2.6	25.8 ± 8.4	254 ± 52	91 ± 25
rtPA-del	13.4 ± 1.7	25.0 v 8.4	299 ± 66	99 ± 32

Table 1. **Hydroxyproline levels in colonic anastomotic tissue and fascia of the abdominal wall on day 7 after operation.**
Hydroxyproline levels are expressed as mean values + SD (n=12) of hydroxyproline concentration (mg/mg dry tissue) and hydroxyproline content (mg/mm tissue).

Experiment 2: ileal anastomoses

Clinical course and survival: As in Experiment 1, the rats became severely ill and body weight decreased during the three days of this experiment. No differences were observed in the course of body weight. The survival rate was 100%.

Anastomotic strength and fascial wound strength: No differences between the groups were observed in anastomotic bursting pressure, breaking strength, or fascial wound strength (Figure 6). Wound hydroxyproline content or histology were not investigated.

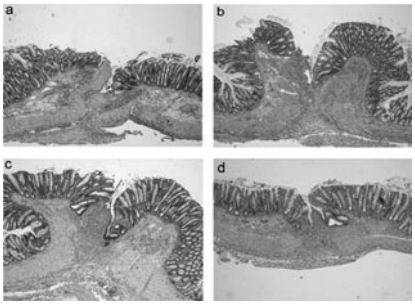

Figure 5. **Anastomotic histology.**
Hematoxylin and eosin-stained sections of colonic anastomoses collected on day 3 (a: controls and b: rtPA group) and day 7 (c: controls and d: rtPA group).

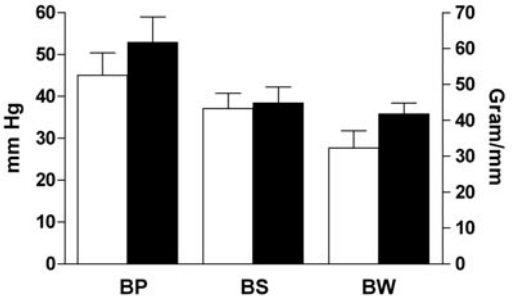

Figure 6. **Wound breaking strength in experiment 2.**
Bursting pressure (BP, left y-axis) and breaking strength (BS, right y-axis) for ileal anastomoses and fascial breaking strength at day 3 after operation. Black bars represent rtPA group, white bars represent controls. Data are depicted as mean + SEM (n=14).

DISCUSSION

The current study demonstrates that rtPA in a dosage sufficient to decrease the rate of intra-abdominal abscesses in a peritonitis model does not affect the healing of colon and small bowel anastomoses nor the abdominal fascial incision. Intra-abdominal abscesses are a well-recognized source of abdominal sepsis, hence decreasing or preventing abscess formation should be an an important aim of treatment(2;6). Fibrin deposition is an integral part of the host defense mechanism and a major factor in the formation of abscesses (19), but it is also intrinsic to the normal wound healing sequence (20).

Therefore intraperitoneal application of a fibrin-specific(12) plasminogen activator such as rtPA has regained attention as an adjunct to the treatment of peritonitis to prevent residual or recurrent abscesses (10;11;14;21). This line of research, however, has not as of yet led to intraperitoneal application of fibrinolytic drugs in the clinical setting because clinicians fear impairment of wound healing and the possibility of bleeding associated with intra-abdominal fibrinolysis.

A general conclusion regarding efficacy and safety of rtPA in the context of wound healing can not be drawn from reported experimental studies, because these studies differ in design, dosage, and the form of administration of tPA. Evans et al. showed in a non-infectious peritoneal ischemia model of adhesion formation that the concentration of rtPA in 0.9% NaCl needed to decrease formation of adhesions disrupts early wound healing of the abdominal fascia (13). These findings seem to be in contrast to those in the present study, where wound healing remained unaffected even after a 1.5-fold greater intraperitoneal dose of rtPA (altogether 3.75 mg during 24 h). This apparent discrepancy may be due to high levels of plasminogen activator inhibitors (PAI) which are supposedly present in our model. Increased PAI levels present during peritonitis in both the abdominal compartment and plasma result in less plasminogen activator activity (PAA) than might be expected on the basis of the dose given(8;22-24). Houston et al. reported a beneficial effect of rtPA on colonic anastomotic bursting strength in a model of perianastomotic infection(25). A single intraperitoneal dose of 0.04 mg rtPA has been reported to be sufficient to treat a perianastomotic abscess but not to prevent abscesses in a model of generalized peritonitis(26). Menzies and Ellis demonstrated that intraperitoneal application of rtPA in rabbits in a non-infectious adhesion model did not adversely affect the colonic anastomotic healing nor the strength of the abdominal fascial wound(27). RtPA was delivered in a gel formula, which acts as a slow release vehicle, resulting in lesser intraperitoneal concentrations of the fibrinolytic agent. Altogether, the data now available, including those from the present, more comprehensive study, suggest that exogenous tPA, in a dose which prevents abscess formation, does not impair wound healing of intestinal anastomoses and the abdominal wall fascia.

Apart from the possible detrimental effects on wound repair, a second major concern of intraperitoneal fibrinolytic therapy is the risk of local and systemic bleeding. As stated before, Van Goor et al. demonstrated that local as well as systemic PAA is decreased during peritonitis. As a consequence, the risk of bleeding after administration of rtPA was considered to be low(22;28). In the current study only one rat that died prematurely exhibited signs of bleeding, whereas all other animals did not, thereby suggesting that bleeding manifestations after rtPA administration are unlikely.

The dose scheme used in the rtPA group was developed in prior studies in our laboratory(10;14). A five-fold decrease in the original dose used by us (such as now used in the rtPA-low group) is still effective in decreasing abscess formation and may be applied when prolonged treatment is desirable. Also, a decrease in abscess formation can still be achieved even if the first rtPA dose is delayed for 24 hours (Buyne et al. unpublished data). This time frame was chosen (and applied in the current rtPA-del group), because treatment of patients with an acute abdomen often starts some time after onset of peritonitis(29). Furthermore, it should also be emphasized that all rats in the present experiment received antibiotics and developed abscesses, if rtPA treatment is omitted.

RtPA treatment does not weaken the bursting strength of anastomoses in either small or large bowel. Whereas anastomosing the small bowel is well-accepted in patients with severe peritonitis, many surgeons are reluctant to perform primary, large bowel anastomosis. In the recent literature however, a plea is made for constructing a primary anastomosis in treating perforated diverticulitis, a common disorder that could benefit from rtPA treatment(30-32). This interest explains the focus of the present study on healing of a colonic anastomosis, which we investigated comprehensively. The results were confirmed for a small bowel anastomosis in a study of limited size. For the latter, the rtPA regimen was chosen which we believe to constitute the highest risk: the highest dose investigated as administered immediately after operation. Also, analysis was limited to the 3rd postoperative day, where wound strength is at its nadir and wounds are most vulnerable.

The current animal model was developed to study different aspects of peritonitis and especially the pathophysiology and treatment modalities of intra-abdominal abscesses(15). Our model, which represents many of the important features of secondary peritonitis, has proven to be reproducible with respect to the local cellular response, cytokine expression, and abscess formation(10;11;14) This model has successfully been used to stepwise investigate the efficacy of rtPA in decreasing abscess formation and has demonstrated that systemic antibiotic therapy alone has no added value in decreasing the rate of abscess formation(11). In the current study, fascial and anastomotic healing, recognized as an important aspect of operative treatment of peritonitis, appears to be unaffected by fibrinolytic treatment. The results of the sequential studies using this model justify clinical research on the safety and efficacy of intraperitoneal application of rtPA.

Acknowledgement

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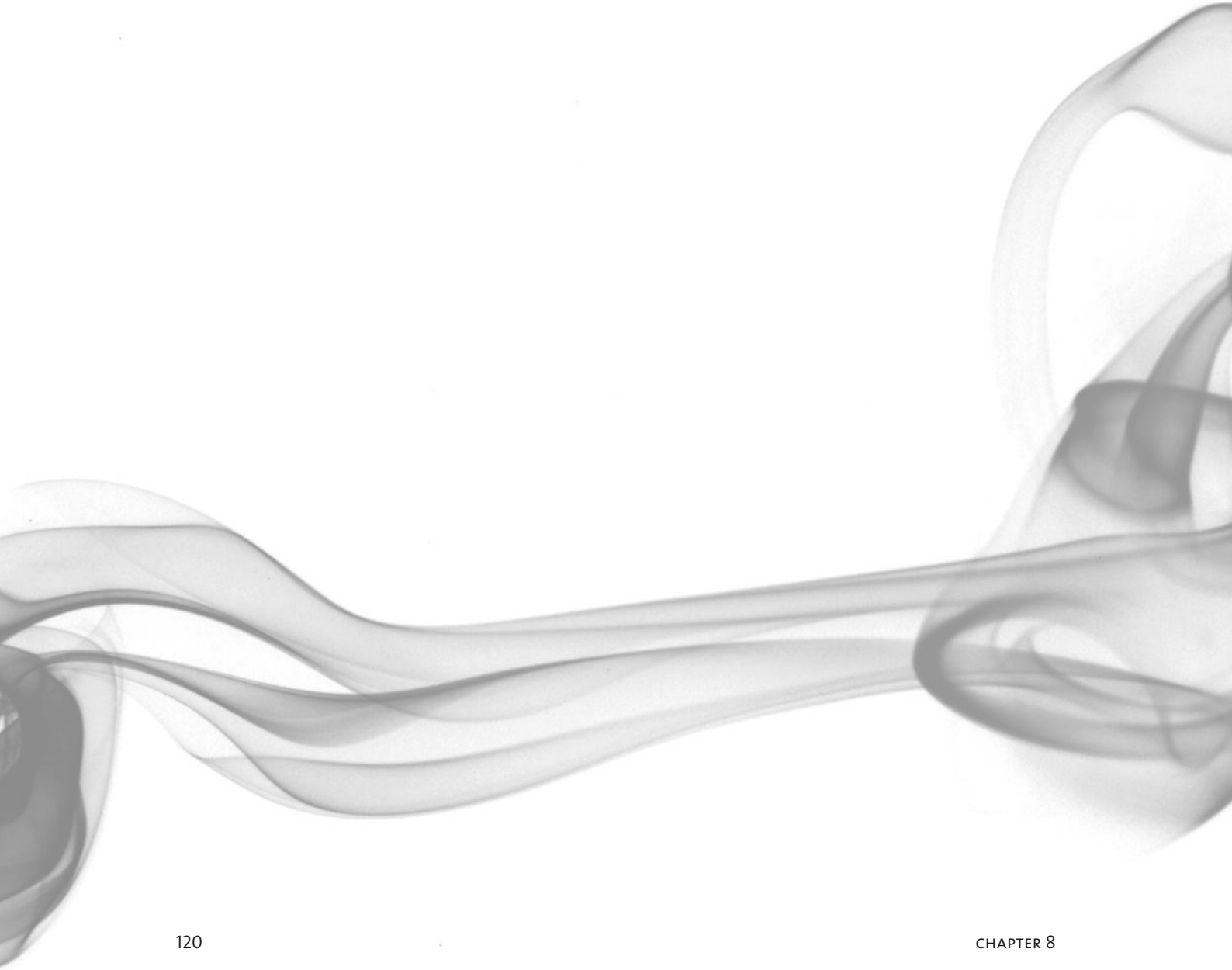
REFERENCE LIST

(1) Farthmann EH, Schoffel U. Epidemiology and pathophysiology of intraabdominal infections (IAI). Infection 1998 Sep;26(5):329-34.
(2) Marshall JC. Intra-abdominal infections. Microbes Infect 2004 Sep;6(11):1015-25.
(3) Wong PF, Gilliam AD, Kumar S, Shenfine J, O'Dair GN, Leaper DJ. Antibiotic regimens for secondary peritonitis of gastrointestinal origin in adults. Cochrane Database Syst Rev 2005;(2):CD004539.
(4) Vermeulen J, Akkersdijk GP, Gosselink MP, Hop WC, Mannaerts GH, van der HE, et al. Outcome after emergency surgery for acute perforated diverticulitis in 200 cases. Dig Surg 2007;24(5):361-6.
(5) Breitenstein S, Kraus A, Hahnloser D, Decurtins M, Clavien PA, Demartines N. Emergency left colon resection for acute perforation: primary anastomosis or Hartmann's procedure? A case-matched control study. World J Surg 2007 Nov;31(11):2117-24.
(6) Marshall JC, Innes M. Intensive care unit management of intra-abdominal infection. Crit Care Med 2003 Aug;31(8):2228-37.
(7) Schein M. Surgical management of intra-abdominal infection: is there any evidence? Langenbecks Arch Surg 2002 Apr;387(1):1-7.
(8) Ince A, Eroglu A, Tarhan O, Bulbul M. Peritoneal fibrinolytic activity in peritonitis. Am J Surg 2002 Jan;183(1):67-9.
(9) Reijnen MM, Bleichrodt RP, van GH. Pathophysiology of intra-abdominal adhesion and abscess formation, and the effect of hyaluronan. Br J Surg 2003 May;90(5):533-41.
(10) Buyne OR, van Goor H, Bleichrodt RP, Verweij PE, Hendriks T. Both tissue-type plasminogen activator and urokinase prevent intraabdominal abscess formation after surgical treatment of peritonitis in the rat. Surgery 2008 Jul;144(1):66-73.
(11) Buyne OR, Bleichrodt RP, van Goor H, Verweij PE, Hendriks T. Plasminogen activator, but not systemic antibiotic therapy, prevents abscess formation in an experimental model of secondary peritonitis. Br J Surg 2008 Oct;95 (10):1287-93.
(12) Li WY, Chong SS, Huang EY, Tuan TL. Plasminogen activator/plasmin system: a major player in wound healing? Wound Repair Regen 2003 Jul;11(4):239-47.
(13) Evans DM, McAree K, Guyton DP, Hawkins N, Stakleff K. Dose dependency and wound healing aspects of the use of tissue plasminogen activator in the prevention of intra-abdominal adhesions. Am J Surg 1993 Feb;165(2):229-32.
(14) Buyne OR, Bleichrodt RP, van Goor H, Verweij PE, Hendriks T. Tissue-type plasminogen activator prevents formation of intra-abdominal abscesses after surgical treatment of secondary peritonitis in a rat model. Int J Colorectal Dis 2006 Nov 7;.
(15) Buyne OR, Bleichrodt RP, Verweij PE, Groenewoud HM, van Goor H., Hendriks T. A peritonitis model with low mortality and persisting intra-abdominal abscesses. Int J Exp Pathol 2006 Oct;87(5):361-8.
(16) Hendriks T, Mastboom WJ. Healing of experimental intestinal anastomoses. Parameters for repair. Dis Colon Rectum 1990 Oct;33(10):891-901.

(17) Posma LA, Bleichrodt RP, van GH, Hendriks T. Ischemia and prolonged reperfusion before anastomotic construction do not reduce wound strength in the rat intestine. Surgery 2006 May;139(5):671-7.
(18) Ikeda M, Sorimachi K, Akimoto K, Yasumura Y. Reversed-phase high-performance liquid chromatographic analysis of hydroxyproline and proline from collagen by derivatization with dabsyl chloride. J Chromatogr 1993 Nov 24;621(2):133-8.
(19) Rotstein OD. Role of fibrin deposition in the pathogenesis of intraabdominal infection. Eur J Clin Microbiol Infect Dis 1992 Nov;11(11):1064-8.
(20) Laurens N, Koolwijk P, de Maat MP. Fibrin structure and wound healing. J Thromb Haemost 2006 May;4(5):932-9.
(21) van Veen SQ, Meijers JC, Levi M, van Gulik TM, Boermeester MA. Effects of intra-abdominal administration of recombinant tissue plasminogen activator on coagulation, fibrinolysis and inflammatory responses in experimental polymicrobial peritonitis. Shock 2007 May;27(5):534-41.
(22) van Goor H, de Graaf JS, Grond J, Sluiter WJ, van der MJ, Bom VJ, et al. Fibrinolytic activity in the abdominal cavity of rats with faecal peritonitis. Br J Surg 1994 Jul;81(7):1046-9.
(23) Holmdahl L. The role of fibrinolysis in adhesion formation. Eur J Surg Suppl 1997;(577):24-31.
(24) Haslinger B, Kleemann R, Toet KH, Kooistra T. Simvastatin suppresses tissue factor expression and increases fibrinolytic activity in tumor necrosis factor-alpha-activated human peritoneal mesothelial cells. Kidney Int 2003 Jun;63(6):2065-74.
(25) Houston KA, McRitchie DI, Rotstein OD. Tissue plasminogen activator reverses the deleterious effect of infection on colonic wound healing. Ann Surg 1990 Feb;211(2):130-5.
(26) van Goor H. In vitro studies on the effect of recombinant tissue plasminogen activator on the rat fibrinolytic system. Thesis: Fibrinolytic therapy in generalized peritonitis to prevent intraabdominal abscess formation. Nijmegen: 1996. p. 125-34.
(27) Menzies D, Ellis H. The role of plasminogen activator in adhesion prevention. Surg Gynecol Obstet 1991 May;172(5):362-6.
(28) van Goor H, Bom VJ, van der MJ, Sluiter WJ, Bleichrodt RP. Coagulation and fibrinolytic responses of human peritoneal fluid and plasma to bacterial peritonitis. Br J Surg 1996 Aug;83(8):1133-5.
(29) Hartl WH, Wolf H, Schneider CP, Kuchenhoff H, Jauch KW. Secular trends in mortality associated with new therapeutic strategies in surgical critical illness. Am J Surg 2007 Oct;194(4):535-41.
(30) Abbas S. Resection and primary anastomosis in acute complicated diverticulitis, a systematic review of the literature. Int J Colorectal Dis 2007 Apr;22(4):351-7.
(31) Constantinides VA, Tekkis PP, Athanasiou T, Aziz O, Purkayastha S, Remzi FH, et al. Primary resection with anastomosis vs. Hartmann's procedure in nonelective surgery for acute colonic diverticulitis: a systematic review. Dis Colon Rectum 2006 Jul;49(7):966-81.
(32) Trompetas V. Emergency management of malignant acute left-sided colonic obstruction. Ann R Coll Surg Engl 2008 Apr;90(3):181-6.



Chapter 8

Summary and general discussion

SUMMARY

Secondary peritonitis is a serious condition bearing significant morbidity and mortality. Improvements have been made in the standard therapy for peritonitis, consisting of surgical source control and debridement, systemic antibiotic treatment and supportive (intensive) care. Yet, mortality and morbidity have only marginally improved. In case of complications such as an intra-abdominal abscess, the prognosis becomes even worse. These abscesses are well-known causes of recurrent or ongoing abdominal infection. The deposition of fibrin is a key step in abscess formation. Breaking down fibrin or preventing its formation is an obvious goal in order to combat abscesses.
This thesis focuses upon the feasibility and efficacy of intraperitoneal administration of recombinant tissue-type plasminogen activator (rtPA) to reduce abscess formation in a rat model of secondary peritonitis.

Chapter 1 describes the three major physiologic defense mechanisms of the abdominal cavity against invasion of micro-organisms. I. Bacterial clearance via diaphragmatic stomata is achieved by cephalad directed stream of peritoneal fluid. II. Upon peritoneal contamination local inflammatory cells, i.e. macrophages and mesothelial cells, are activated and recruited. Cytokines and chemokines are expressed to further enhance the local inflammatory response. Permeability of local vessels increases leading to formation of an exudate containing chemokines and cytokines. III. Both intra-abdominal fibrinolysis and coagulation are upregulated, the latter predominating in the early phase. As a consequence fibrin deposition occurs in order to contain or compartmentalize the focus of infection. Next, factors are described that interfere with physiologic resolution of peritonitis. Fibrin adhesions promote abscess formation and hamper bacterial clearance via the diaphragmatic stomata. Bacterial synergy will further adversely influence the outcome of peritonitis.
The presence of adjuvant substances such as bile or blood impede the local inflammatory response. In the second section, the molecular properties of fibrin are described as well as its formation and degradation. In the abdominal cavity tissue factor is expressed predominantly by mesothelial cells as a result of contamination or infection. Subsequently, the extrinsic pathway of coagulation is activated via factor V, VII and X. Next, thrombin is activated and converts fibrinogen into fibrin. A fibrin mesh is formed and subsequently factor XIIIa further stabilizes the fibrin mesh by cross-linking the fibrin molecules.

Physiologic break down of fibrin occurs by transformation of the pro-enzyme plasminogen into its active from plasmin by plasminogen activators (Pas). Two types of PAs are recognized: urine-type PA (u-PA) and tissue-type PA (tPA), only the latter being relevant in the abdominal cavity. Resolution of fibrinolysis is achieved by counteraction of tPA by plasminogen activator inhibitor (PAI). Human mesothelial cells play a critical role in the process of both coagulation and fibrinolysis since procoagulatory TF, fibrinolytic tPA as well as PAI are expressed by these cells.

Chaper 2 reports on the development of a model suitable to study several important features of secondary peritonitis, in particular the formation of abscesses and the local inflammatory response. Fifty male Wistar rats were injected intraperitoneally with a mixture of sterile rat feces, increasing doses of E. coli (10^4-10^8 cfu/ml) and a fixed dose of B. Fragilis (10^4 cfu/ml) thereby mimicking an intestinal perforation. Ten rats were injected with sterile rat feces only, and served as a control group. In all rats, a laparotomy was performed after one hour and the peritoneal cavity was debrided. Blood samples were taken after 6 and 24 hours, in order to perform bacterial culturing and to determine plasma levels of IL-6 and TNFα. Abdominal fluid samples were collected after 24 and 72 hours. Local peritoneal levels of the cytokines IL-1β, IL-10-, IL-6 and TNFα were determined and cell counts were performed. The rats were killed after 5 days by CO2 asphyxation and the abdomen was inspected for abscesses.
Mortality was 90% in the groups receiving the two highest doses of bacteria and 30% in the remaining groups. In the latter groups all surviving rats but one showed intra-abdominal abscesses and bacteremia was encountered frequently, especially after 24 hours in the 10^5 cfu E. coli group. No systemic levels of TNFα were detected and plasma IL-6 concentrations were elevated after 6 hours and significantly lowered after 24 hours. Generally speaking, peritoneal cytokine levels were elevated in experimental groups as well as in controls after 24 hours and declined after 72 hours. The groups receiving the highest dose of bacteria had the highest levels of cytokines. The cellular response did not differ between groups. The number of neutrophils was elevated at 24 hours and steadily declined in time. Eosinophils increased and monocytes remained relatively stable throughout the experiment. It was concluded that the present model for secondary peritonitis has provided satisfactory results regarding abscess formation, survival and local inflammatory response. The model is suitable to study the mechanisms involved in intra-abdominal abscess formation after surgical treatment of generalized peritonitis.

In **chapter 3** the model described in the preceding chapter was used to study the effect of intra-abdominally applied rtPA on abscess formation. In previous experimental studies, rtPA was found to be effective in reducing abscess formation in rodent models of peritonitis without any apparent increase in bleeding complications. However, in those studies intra-abdominal application of rtPA was associated with considerable and significantly increased mortality, probably as a result of release of bacteria from the peritoneal cavity, with subsequent bacteriaemia, sepsis and/or an exaggarated local immune response. The present study was initiated to determine whether the effect of intra-abdominal application of rtPA can be optimized by changing the method of application and the dosage scheme. In addition, it was studied whether intraperitoneal rtPA therapy is associated with an increased inflammatory response in the abdominal cavity, increased incidence and severity of bacteremia or mortality.
Fifty-five animals were randomly divided into three groups of 14 and one group of 13 rats. Bacterial peritonitis was induced as described in chapter 2 and surgical therapy was performed likewise. Three experimental groups received intraperitoneal injections with 1.25 mg of rtPA at 1 h (rtPA1), 1 h and 6 h (rtPA2) and 1, 6 and 24 h (rtPA3) after inoculation, respectively. The animals in the fourth group (control) received sterile saline only at each time point. Blood samples for culture were taken at 6 and 24 hours after inoculation, peritoneal fluid samples for cytokines and cell counts were taken after 24 hours. After 5 days the animals were killed by CO_2 asphyxiation. The abdomen was inspected, with particular attention to the number and location of abscesses.
RtPA treatment significantly reduced abscess formation. Animals in group rtPA3 had no abscesses in contrast to 88% of the controls. In the rtPA1 and rtPA2 group, frequency of abscess formation was 58 and 33%, respectively. Mortality, course of body weight and bacteremia were not affected by rtPA and neither were peritoneal cell counts and levels of TNF-α, IL-1β, IL-6 and IL-10. No bleeding complications were observed.
The conclusion was that rtPA reduces intra-abdominal abscess formation after surgical treatment of generalized peritonitis without increasing mortality or affecting the local inflammatory response.

In **chapter 4** a comparison was made between two different fibrinolytic agents in a larger experiment. Again, peritonitis was induced in 80 male Wistar rats as described earlier.
The animals were divided into four groups of twenty rats each. Two experimental groups were treated by intra-abdominal administration of a fibrinolytic agent: one group was treated with three doses of

1.25 mg rtPA at 1, 6 and 24 hours after inducing peritonitis, a second experimental group received three doses of 725.000 IU of urokinase. One control group was treated with sterile saline only. A second control group served as a negative protein control and received 725.000 IU of streptokinase, which does not affect the rat coagulatory system. Blood samples were taken for culture at 6 and 24 hours after inducing peritonitis and abdominal fluid samples were taken at 24, 72 and 120 hours for cell counts and to determine levels of IL-6, IL-10 and TNFα. After 5 days the animals were sacrificed and the abdomen inspected for the number and location of abscesses.
The data show that both rtPA and urokinase strongly (> 75%) and significantly ($P < .05$) reduced abscess formation without negative side effects. No bleeding complications were observed. Generally speaking, fibrinolytic therapy resulted in less neutrophils and lymphocytes and more macrophages and eosinophils in time but did not essentially alter the courses of IL-6 and IL-10 (decreasing in time) or TNFα (increasing in time) levels.
It was concluded that both rtPA and urokinase effectively and safely reduce abscess formation in a rat model for treatment of secondary peritonitis.

In clinical practice, commencement of adequate therapy may vary due to the time elapsed between onset of peritonitis and presentation of clinical symptoms or because of delay in establishing the diagnosis. In our experiments so far rtPA treatment has been restricted to the first 24 hours after inducing peritonitis. Therefore **chapter 5** addresses the effect of delayed and prolonged treatment with intraperitoneal rtPA. Anticipating the need to maximally reduce the treatment-related risk profile, the effect of lowering the dose was studied as well.
Bacterial peritonitis was induced in male Wistar rats as described before. Surgical debridement was performed after 1 hour. Abdominal fluid samples were taken at 24 and 72 hours after inducing peritonitis for measurements of Il-6, IL-10 and TNFα and cell counts. After 5 days rats were sacrificed the abdomen was inspected for abscesses.
Two experiments were performed. In experiment I, four groups of animals (n=20 each) were treated with rtPA. One group received 1.25 mg of rtPA at 1, 6 and 24 hours (rtPA), in another group rtPA treatment was prolonged: 7 doses in 72 hours (rtPA prol). One group received three doses of rtPA after 24 hour delay (rtPA 24) and another group after 48 hour delay (rtPA 48). There was one control group (n=20) treated with saline at 1, 6 and 24 hours. In experiment II two groups (n=15 each) were treated with three doses of rtPA of either 0.25 mg or 1.25 mg.

Early administration of rtPA in both doses significantly reduced the number of rats with abscesses and the abscess load per rat. Delayed treatment still significantly reduced abscess load but not the incidence of abscesses. No adverse effects of intra-abdominal rtPA treatment were observed. No meaningful differences in local inflammatory response were found. RtPA was most effective when applied early and for 72 hours. However, under those circumstances mortality increased significantly. No clear explanation for this observation was found.
These results corroborate the potential benefits of rtPA as an adjunct to the standard treatment of peritonitis in the clinical setting. Safe rtPA dosages should be tested in a phase II study. In those cases, where treatment for a longer period is needed, the effects of prolonged low dose rtPA treatment should be investigated.

Systemic antibiotic therapy is one of the important elements of the treatment of peritonitis. Until this point antibiotic therapy was not administered in the consecutive studies in order to focus exclusively on the effect of intra-abdominal fibrinolytic therapy.

Chapter 6 describes the effects of systemic antibiotics on intra-abdominal abscess formation and survival, both in the absence and the presence of intraperitoneally applied rtPA.
Seventy-two animals were randomly divided into four equal groups. In all rats peritonitis was induced as described earlier. The first (control) group received no further drug treatment, the second group (rtPA) received three doses of 1.25 mg of rtPA intra-abdominally, the third group systemic antibiotics (AB) and the fourth group antibiotics plus intra-abdominal rtPA (rtPA+AB) group. Antibiotic therapy was given three days and consisted of 15 mg/kg Metronidazol and 15 mg/kg Ceftriaxone twice daily. Blood samples for culture were drawn at 24 hours after inducing peritonitis. Abdominal fluid samples were taken at 24, 72 and 120 hours for cell counts and to determine levels of IL-6, IL-10 and TNFα. As in the preceding experiments the animals were sacrificed after 5 days and the abdomen was inspected for number, size and location of abscesses.
Antibiotics did not significantly affect abscess formation but rtPA significantly reduced the number of rats with abscesses and the abscess load per rat, both in absence and presence of antibiotic therapy. One rat died prematurely. No adverse side effects were observed and no meaningful differences in the local inflammatory response were found.
The conclusion was that the administration of systemic antibiotics does not influence abscess

formation, while intra-abdominal application of rtPA consistently reduces abscess formation. In addition to systemic antibiotic therapy, rtPA is a valuable adjunct in the therapy of peritonitis.

As part of the surgical therapy for peritonitis, often an intestinal anastomosis needs to be constructed or visceral organ repair will be needed otherwise. As shown in the preceding experiments, fibrinolytic therapy reduces intra-abdominal abscesses. On the other hand, fibrin deposition is essential for and intrinsic to early wound repair, providing a provisional scaffold for initial structural wound stability and cell migration. It can be hypothesized that any interference with fibrin formation could lead to wound healing disturbances, particularly so in anastomotic healing.

Chapter 7 addresses the possible effects of the intra-abdominal administration of rtPA on anastomotic healing and healing of the fascial wound of the abdominal wall after surgical treatment for peritonitis in a rat model.
Peritonitis was induced and treated in 148 rats as described earlier. Two experiments were performed. In experiment I, 1.25 mg of rtPA was given intraperitoneally at 1, 6 and 24 hours after inducing peritonitis to one group. A second group was treated likewise but with 0.25 mg of rtPA. A third group received 1.25 mg of rtPA after 24 hour delay. The fourth group served as a control group and received sterile saline at 1, 6 and 24 hours. Each group consisted of 30 rats. After surgical debridement, resection of 2 cm of the descending colon, 3 cm proximal to the peritoneal reflection was performed. Continuity was restored by constructing an end-to-end anastomosis with 8 single-layer, inverting, interrupted sutures. Within each group, half of the animals were killed at 3 and 7 days after operation, respectively. In experiment II, ileal resection and anastomosis was performed immediately after debridement of the abdominal cavity and the animals were randomized into two groups (n=14 each), a rtPA group and a control group. All rats were killed at the third postoperative day. In both experiments abscesses were counted and bursting pressure, breaking strength and hydroxyproline content of anastomoses and abdominal fascial wounds were determined.
Treatment with rtPA in both doses significantly reduced abscess formation. Colonic anastomoses as well as fascial wounds increased in strength from day 3 to day 7. No significant difference between groups were observed with respect to bursting pressure, breaking strength and hydroxyproline content for colonic as well as small bowel anastomoses. It was concluded that rtPA consistently reduces abscesses and does not affect the healing of intestinal anastomoses or the abdominal fascia.

GENERAL DISCUSSION

Introduction

Secondary peritonitis often also described as abdominal sepsis or abdominal infection, is caused by loss of integrity of a hollow viscus and is a life threatening condition(1). Treatment is based on three major principles. The first goal is to achieve source control by surgical intervention, i.e. resection, repair or isolation of the organ involved and debridement. A second aim is timely commencement of appropriate systemic antibiotic therapy. The third important measure is adequate high care support in order to restore and maintain tissue and organ perfusion and oxygenation(2;3). Despite advances made with respect to surgical therapy, antibiotic treatment and intensive care supportive treatment, mortality still is approximately 30%(4).

An intra-abdominal abscess is a known source of recurrent or ongoing abdominal sepsis(5;6). In those patients where an intra-abdominal abscess develops during the course of treatment for secondary peritonitis, mortality may increase up to 60%(7). Abscesses can be treated minimal invasively via percutaneous drainage yielding a success rate of 80% in unilocular abscesses to 30% in more complicated cases(8) However, if this option fails, surgical drainage must be performed. Repeat laparotomy entails a hazard ratio of 2.5 for sepsis related complications and an overall mortality rate of 26%(9). These numbers are even worse in case of urgent relaparotomy(10).

Considering the above, reduction of the formation of intra-abdominal abscesses is an obvious goal in the treatment of secondary peritonitis.

Fibrin

Fibrin is the major component of an abscess wall(11) and therefore reducing the fibrin content of intra-abdominal abscesses was a main subject of this thesis. This can be achieved by two different approaches. One can either interfere with intraperitoneal coagulation or enhance intra-abdominal fibrinolysis.

Anticoagulatory therapy in peritonitis

Attempts have been made to reduce intraperitoneal coagulation by heparin treatment. Bagree et al reported 75% mortality after intraperitoneal adminstration of heparin(12). Controversely, others have reported a reduction of abscesses together with an increased survival after intraperitoneal application

of heparin in experimental peritonitis(13;14). It was shown in several animal models that subcutaneous application of heparin also reduced intra-abdominal abscesses and improved survival(13-19). The working mechanism of heparin in peritonitis is unclear. A possible explanation is that heparin prevents the formation of fibrin and the subsequent entrapment of bacteria within fibrin, thereby rendering the micro-organisms more susceptible to absorption from the peritoneal cavity via the diaphragmatic lacunae and digestion by local peritoneal phagocytes(14). An important drawback on the use of heparin is its unpredictable anticoagulatory effect and the risk of bleeding(20;21). Another disadvantage might be the effect of heparin on wound healing in general. Performing an intestinal anastomosis or repairing an injured organ is often part of the surgical therapy. In cell culture studies, heparin and growth factors are associated with rapid and effective endothelial cell repair (22-24). There are however, no data on the effect of heparin on anastomotic healing.

Fibrinolytic therapy in peritonitis

Tissue-type plasminogen activator (tPA) has been applied in order to reduce abscess formation in experimental peritonitis. Rotstein et al reported a significant reduction of abscesses but also increased mortality after intraperitoneal. administration of tPA. Mortality was reduced after administration of systemic antibiotics(25;26). A similar abscess reducing affect of tPA was reported by van Goor et al, but no correlation between bacteremia and mortality was found(27). Activation of coagulation and formation of fibrin deposits as a result of inflammation is considered instrumental in containing inflammatory activity to the site of injury or infection. Application of fibrinolytics potentially disturbs this process. Bacteria caught in fibrin clots may be released much faster into the abdominal cavity when intra-abdominal fibrinolytics are given, carrying the risk of bacteriaemia and sepsis. It is reasonable to assume that mortality will increase as well, when no systemic antibiotics are given. This is supported by the fact that administration of systemic antibiotics almost completely prevents mortality(28;29). We found no effect of r-tPA on the inflammatory response in the abdominal cavity, whereas a systemic effect is very unlikely since r-tPA will be inactivated by PAI which is present in high concentrations in the systemic circulation.

Bleeding as a result of fibrinolytic therapy is unlikely to occur because of the fact that the systemic plasminogen activator activity (PAA) after intraperitoneal administration of tPA has proven to be low as a result of increased plasminogen activator inhibitor (PAI) levels(30).

The effect of tPA on wound healing, and anastomotic healing in particular, has been subject of

research. The results of studies on this subject are conflicting and the studies quite differ in doses of tPA as well as in the way tPA was delivered(31-33).
In the current literature on peritonitis, data are available on drugs which could be considered as alternatives to tPA. There is one case report on the succesful use of urokinase for relapsing peritonitis during the course of peritoneal dialysis(34). Studies on the application of polyanionic polysaccharides yield equivocal results. Reijnen et al. have reported a significant reduction of abscesses after intra-peritoneal adminstration of hyaluronic acid (HA) and carboxymethyl cellulose (CMC)(24;35;36) ,while Sikkink et al. found no reduction in abscess formation(37). Bae et al. have shown that polysaccharides isolated from Phellinus mushrooms also significantly reduce abscesses(38;39). Several experimental studies indicated that polysaccharides do not affect anastomotic strength(40-44), but in one study CMC was associated with an increased rate of perianastomotic abscess(45). In a randomized clinical trial regarding to the effect of polysaccharides on anastomotic healing, it was found that CMC adversely affects the sequelae of anastomotic leakage(46).

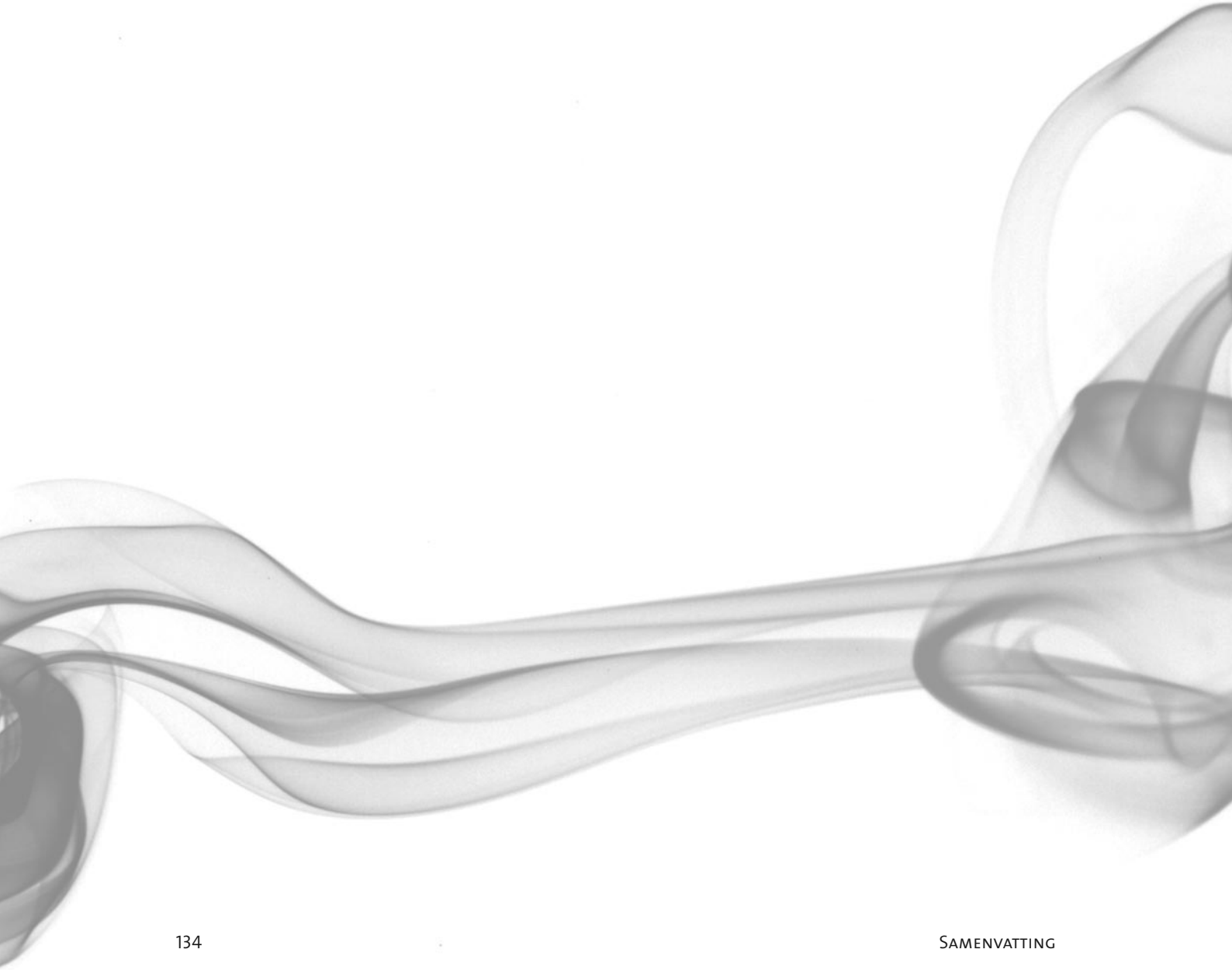
The ultimate goal of any experimental research would be to employ the results in clinical practice. In this dissertation several clinically relevant issues have been addressed. The feasibility and efficacy of intra-abdominal administration of rtpA in order to reduce abscess formation was demonstrated. RtPA repeatedly and consistently reduced the number of abscesses as well as the abscess load after surgical treatment for peritonitis. Intra-abdominal fibrinolytic therapy does not interfere with the local inflammatory response and does not increase the risk of bleeding. The incidence and severity of bacteremia was not affected by rtPA. Systemic antibiotic therapy has no additional value with respect to the reduction of intra-abdominal abscess formation. Furthermore anastomotic healing as well as healing of the abdominal fascial wound were not affected.
Based on these findings clinical research on the utilisation of intra-abdominal recombinant tissue-type plasminogen activator is warranted.

REFERENCE LIST

(1) Farthmann EH, Schoffel U. Epidemiology and pathophysiology of intraabdominal infections (IAI). Infection 1998 Sep;26(5):329-34.
(2) Chong AJ, Dellinger EP. Current treatment of intraabdominal infections. Surg Technol Int 2005;14:29-33.
(3) Polk HC, Jr. Generalized peritonitis: a continuing challenge. Surgery 1979 Nov;86(5):777-8.
(4) Holzheimer RG, Dralle H. Paradigm change in 30 years peritonitis treatment -- a review on source control. Eur J Med Res 2001 Apr 20;6(4):161-8.
(5) Fry DE, Clevenger FW. Reoperation for intra-abdominal abscess. Surg Clin North Am 1991 Feb;71(1):159-74.
(6) Rotstein OD. Role of fibrin deposition in the pathogenesis of intraabdominal infection. Eur J Clin Microbiol Infect Dis 1992 Nov;11(11):1064-8.
(7) Marshall JC. Intra-abdominal infections. Microbes Infect 2004 Sep;6(11):1015-25.
(8) vanSonnenberg E, Wittich GR, Goodacre BW, Casola G, D'Agostino HB. Percutaneous abscess drainage: update. World J Surg 2001 Mar;25(3):362-9.
(9) Lamme B, Boermeester MA, Belt EJ, van Till JW, Gouma DJ, Obertop H. Mortality and morbidity of planned relaparotomy versus relaparotomy on demand for secondary peritonitis. Br J Surg 2004 Aug;91(8):1046-54.
(10) Unalp HR, Kamer E, Kar H, Bal A, Peskersoy M, Ali OM. Urgent abdominal re-explorations. World J Emerg Surg 2006;1:10.
(11) Rotstein OD. Role of fibrin deposition in the pathogenesis of intraabdominal infection. Eur J Clin Microbiol Infect Dis 1992 Nov;11(11):1064-8.
(12) Bagree MM, Aneja RK. Adjuvant effect of heparin on peritonitis induced in rats. Jpn J Surg 1983 Nov;13(6):546-8.
(13) O'Leary JP, Malik FS, Donahoe RR, Johnston AD. The effects of a minidose of heparin on peritonitis in rats. Surg Gynecol Obstet 1979 Apr;148(4):571-5.
(14) Hau T, Simmons RL. Heparin in the treatment of experimental peritonitis. Ann Surg 1978 Mar;187(3):294-8.
(15) Vela AR, Littleton JC, O'Leary JP. The effects of minidose heparin and low molecular weight heparin on peritonitis in the rat. Am Surg 1999 May;65(5):473-7.
(16) Woltmann A, Kattenbeck K, Broll R, Lebeau A, Gatermann S, Bruch HP. [Does heparin modify the course of chronic abscess-forming peritonitis in the animal model?]. Langenbecks Arch Chir 1997;382(2):107-10.
(17) Gupta S, Jain PK. Low-dose heparin in experimental peritonitis. Eur Surg Res 1985;17(3):167-72.
(18) Chalkiadakis G, Kostakis A, Karayannacos PE, Giamarellou H, Dontas I, Sakellariou I, et al. The effect of heparin upon fibrinopurulent peritonitis in rats. Surg Gynecol Obstet 1983 Sep;157(3):257-60.
(19) Prinz RA, Shuber Z, Fareed J, Bird T, Sandberg L. Effect of heparin and heparin fractions on experimental abscess formation. Arch Surg 1986 Oct;121(10):1173-6.
(20) Magee KD, Campbell SG, Moher D, Rowe BH. Heparin versus placebo for acute coronary syndromes. Cochrane Database Syst Rev 2008;(2):CD003462.

(21) Crowther MA, Warkentin TE. Bleeding risk and the management of bleeding complications in patients undergoing anticoagulant therapy: focus on new anticoagulant agents. Blood 2008 May 15;111(10):4871-9.
(22) Fan SQ, Cai JL, Qin LY, Wang ZH, Liu ZZ, Sun ML. Effect of heparin on production of transforming growth factor (TGF)-beta1 and TGF-beta1 mRNA expression by human normal skin and hyperplastic scar fibroblasts. Ann Plast Surg 2008 Mar;60(3):299-305.
(23) Yao C, Markowicz M, Pallua N, Noah EM, Steffens G. The effect of cross-linking of collagen matrices on their angiogenic capability. Biomaterials 2008 Jan;29(1):66-74.
(24) Galvan L. Effects of heparin on wound healing. J Wound Ostomy Continence Nurs 1996 Jul;23(4):224-6.
(25) Rotstein OD, Kao J. Prevention of intra-abdominal abscesses by fibrinolysis using recombinant tissue plasminogen activator. J Infect Dis 1988 Oct;158(4):766-72.
(26) Rosenthal GA, Quinto J, Kao J, Rotstein OD. Prevention of intra-abdominal abscesses with fibrinolytic agents. Can J Surg 1988 Mar;31(2):98-100.
(27) van Goor H, de Graaf JS, Kooi K, Sluiter WJ, Bom VJ, van der MJ, et al. Effect of recombinant tissue plasminogen activator on intra-abdominal abscess formation in rats with generalized peritonitis. J Am Coll Surg 1994 Oct;179(4):407-11.
(28) Buyne OR, Bleichrodt RP, van GH, Verweij PE, Hendriks T. Plasminogen activator, but not systemic antibiotic therapy, prevents abscess formation in an experimental model of secondary peritonitis. Br J Surg 2008 Oct;95(10):1287-93.
(29) van Goor H, de Graaf JS, Kooi K, Bleichrodt RP. Gentamycin reduces bacteremia and mortality rates associated with the treatment of experimental peritonitis with recombinant tissue plasminogen activator. J Am Coll Surg 1995 Jul;181(1):38-42.
(30) van Goor H, Bom VJ, van der MJ, Sluiter WJ, Geerards S, van der SW, et al. Pharmacokinetics of human recombinant tissue-type plasminogen activator, administered intra-abdominally, in a rat peritonitis model. Eur Surg Res 1996 Jul;28(4):287-94.
(31) Evans DM, McAree K, Guyton DP, Hawkins N, Stakleff K. Dose dependency and wound healing aspects of the use of tissue plasminogen activator in the prevention of intra-abdominal adhesions. Am J Surg 1993 Feb;165(2):229-32.
(32) Menzies D, Ellis H. The role of plasminogen activator in adhesion prevention. Surg Gynecol Obstet 1991 May;172(5):362-6.
(33) Houston KA, McRitchie DI, Rotstein OD. Tissue plasminogen activator reverses the deleterious effect of infection on colonic wound healing. Ann Surg 1990 Feb;211(2):130-5.
(34) Duch JM, Yee J. Successful use of recombinant tissue plasminogen activator in a patient with relapsing peritonitis. Am J Kidney Dis 2001 Jan;37(1):149-53.
(35) Reijnen MM, Meis JF, Postma VA, van GH. Prevention of intra-abdominal abscesses and adhesions using a hyaluronic acid solution in a rat peritonitis model. Arch Surg 1999 Sep;134(9):997-1001.
(36) Reijnen MMPJ, Skrabut EM, Postma VA, Burns JW, van Goor H. Polyanionic Polysaccharides Reduce Intra-abdominal Adhesion and Abscess Formation in a Rat Peritonitis Model. Journal of Surgical Research 2001 Dec;101(2):248-53.

(37) Sikkink CJ, de MB, Bleichrodt RP, van GH. Auto-cross-linked hyaluronic acid gel does not reduce intra-abdominal adhesions or abscess formation in a rat model of peritonitis. J Surg Res 2006 Dec;136(2):255-9.
(38) Bae JS, Ahn SJ, Yim H, Jang KH, Jin HK. Prevention of intraperitoneal adhesions and abscesses by polysaccharides isolated from Phellinus spp in a rat peritonitis model. Ann Surg 2005 Mar;241(3):534-40.
(39) Bae JS, Jang KH, Jin HK. Comparison of intraperitoneal anti-adhesive polysaccharides derived from Phellinus mushrooms in a rat peritonitis model. World J Gastroenterol 2005 Feb 14;11(6):810-6.
(40) Erturk S, Yuceyar S, Temiz M, Ekci B, Sakoglu N, Balci H, et al. Effects of hyaluronic acid-carboxymethylcellulose anti-adhesion barrier on ischemic colonic anastomosis: an experimental study. Dis Colon Rectum 2003 Apr;46(4):529-34.
(41) van Oosterom FJ, van Lanschot JJ, Oosting J, Obertop H. Hyaluronic acid/carboxymethylcellulose membrane surrounding an intraperitoneal or subcutaneous jejunojejunostomy in rats. Eur J Surg 2000 Aug;166(8):654-8.
(42) Reijnen MM, de Man BM, Hendriks T, Postma VA, Meis JF, van GH. Hyaluronic acid-based agents do not affect anastomotic strength in the rat colon, in either the presence or absence of bacterial peritonitis. Br J Surg 2000 Sep;87(9):1222-8.
(43) Hadaegh A, Burns J, Burgess L, Rose R, Rowe E, LaMorte WW, et al. Effects of hyaluronic acid/carboxymethylcellulose gel on bowel anastomoses in the New Zealand white rabbit. J Gastrointest Surg 1997 Nov;1(6):569-75.
(44) Medina M, Paddock HN, Connolly RJ, Schwaitzberg SD. Novel antiadhesion barrier does not prevent anastomotic healing in a rabbit model. J Invest Surg 1995 May;8(3):179-86.
(45) Bowers D, Raybon RB, Wheeless CR, Jr. Hyaluronic acid-carboxymethylcellulose film and perianastomotic adhesions in previously irradiated rats. Am J Obstet Gynecol 1999 Dec;181(6):1335-7.
(46) Beck DE, Cohen Z, Fleshman JW, Kaufman HS, van GH, Wolff BG. A prospective, randomized, multicenter, controlled study of the safety of Seprafilm adhesion barrier in abdominopelvic surgery of the intestine. Dis Colon Rectum 2003 Oct;46(10):1310-9.



Samenvatting

SAMENVATTING

Secundaire peritonitis is een ernstige ziekte die gepaard gaat met aanzienlijke morbiditeit en mortaliteit. Er is vooruitgang geboekt in de standaardbehandeling van peritonitis, die bestaat uit chirurgisch debridement, dan wel isoleren van de bron van infectie, het toedienen van systemische antibiotica en recuscitatie en intensive care behandeling. Desondanks zijn mortaliteit en morbiditeit slechts weinig verbeterd. Wanneer er een complicatie optreedt gedurende de behandeling, zoals bijvoorbeeld een intra-abdominaal abces, wordt de prognose slechter. Deze abcessen zijn een bekende oorzaak van recidiverende of voortschrijdende abdominale infectie.
De vorming van fibrine is een belangrijke stap in het ontstaan van een abces. Het afbreken van fibrine of het voorkomen van fibrinevorming is derhalve een voor de hand liggend doel om de ontwikkeling van abcessen tegen te gaan.
Dit proefschrift richt zich op de haalbaarheid en efficiëntie van intraperitoneale toediening van recombinant tissue-type plasminogene activator (rtPA) om abcesvorming in een rattenmodel voor secundaire peritonitis te voorkomen.

Hoofstuk 1 beschrijft de drie belangrijkste fysiologische afweermechanismen van de buikholte tegen invadering door micro-organismen: I. Klaring van bacteriën via stomata in het diafragma als gevolg van de craniaalwaarts gerichte circulatie van peritoneaal vocht. II. Na contaminatie van het peritoneum worden locale ontstekingscellen, zoals macrofagen en mesotheelcellen, gerekruteerd en geactiveerd. Cytokines en chemokines komen tot expressie en versterken de locale inflammatoire respons. De doorlaatbaarheid van de locale vaten neemt toe, wat leidt tot een fibrinogeenrijk exsudaat dat chemokines en cytokines bevat. III. Zowel intra-abdominale fibrinolyse als coagulatie zijn geactiveerd, waarbij de laatste de overhand heeft in de vroege fase. Als gevolg hiervan zal zich fibrine vormen, met als doel om het focus van infectie af te grenzen.
Hierna worden in dit hoofdstuk factoren beschreven die interfereren met de fysiologische reactie van de buikholte op de peritonitis. Fibrineuze adhesies bevorderen de vorming van abcessen en verhinderen de bacteriële klaring via de stomata van het diafragma. Bacteriële synergie zal de uitkomst van peritonitis nog verder negatief beïnvloeden. De aanwezigheid van adjuvante stoffen zoals gal of bloed verstoren de locale inflammatoire respons.

In het tweede deel van hoofdstuk 1 worden de vorming en afbraak, alsmede de moleculaire eigenschappen van fibrine beschreven. Als gevolg van contaminatie of infectie wordt in de buikholte tissue factor (TF) tot expressie gebracht. Dit gebeurt voornamelijk door de mesotheelcellen. Vervolgens wordt de extrinsieke stollingsroute geactiveerd via respectievelijk factor V,VII en X. Hierna wordt thrombine geactiveerd en hierdoor wordt fibrinogeen omgezet in fibrine. Een fibrineus netwerk wordt gevormd en vervolgens verder gestabiliseerd door factor XIIIa die de vorming van dwarsverbindingen tussen de fibrine moleculen induceert. De fysiologische afbraak van fibrine gebeurt door plasmine, dat ontstaat na omzetting van het pro-enzym plasminogeen in zijn actieve vorm, door plasminogeen activatoren (PAs). Er zijn twee type PAs bekend: urine-type PA (u-PA) en tissue-type PA (tPA), waarbij de laatste relevant is in de buikholte. Fysiologisch wordt de fibrinolyse beëindigd door het tegengaan van de werking van tPA door plasminogeen activator inhibitor (PAI). De mesotheelcellen spelen een sleutelrol in zowel stolling als fibrinolyse van de buikholte, omdat het procoagulatoire TF, het fibrinolytisch werkende tPA, maar ook PAI door deze cellen tot expressie worden gebracht.

Hoofdstuk 2 beschrijft de ontwikkeling van een model wat geschikt is om verschillende kenmerken van secundaire peritonitis te bestuderen, in het bijzonder de vorming van abcessen en de locale inflammatoire respons. Vijftig mannelijke Wistar ratten werden intraperitoneaal geïnjecteerd met een mengsel van gesteriliseerde rattenfeces, oplopende doses E. Coli (10^4-10^8 cfu/ml) en een vaste dosis B. Fragilis (10^4 cfu/ml). Op deze wijze werd een intestinale perforatie nagebootst. Tien ratten werden geïnjecteerd met alleen steriele feces en dienden als controlegroep. In alle ratten werd na één uur een laparotomie uitgevoerd, waarbij de peritoneaalholte werd ontdaan van avitaal en geïnfecteerd weefsel. Na 6 en 24 uur werden bloedmonsters genomen voor bacteriologische kweken en om plasmaspiegels van IL-6 en TNF-α te bepalen. Monsters van buikvocht werden genomen op 24 en 72 uur na inductie van peritonitis. Hierin werden spiegels van IL1-β, IL-10, IL-6 en TNF-α bepaald en celtellingen uitgevoerd. De ratten werden opgeofferd na 5 dagen door CO_2-asphyxie en de buikholte werd geïnspecteerd op de aanwezigheid van abcessen.
De sterfte bedroegt 90% in de groepen die de hoogste dosis bacteriën toegediend kregen en 30% in de overige groepen. In de laatste groepen werden in alle overlevende ratten intra-abdominale abcessen aangetroffen en was er sprake van een bacteriëmie, met name na 24 uur in de 10^5 cfu E. coli groep. In het bloed was geen TNF-α meetbaar; plasmaspiegels van IL-6 waren verhoogd na 6 uur en daalden significant na 24 uur.

In het algemeen gesteld waren de cytokinespiegels in het buikvocht verhoogd, in zowel de experimentele groepen als in de controles na 24 uur. Deze spiegels namen af na 72 uur. De groepen die de hoogste dosis bacteriën toegediend hadden gekregen, vertoonden ook de hoogste cytokineconcentraties. De cellulaire reactie verschilde niet significant tussen de groepen. Het aantal neutrofielen was verhoogd na 24 uur en daalde gestaag gedurende het experiment. Het aantal eosinofielen steeg en het monocytengetal bleef relatief stabiel.
De conclusie was dat het huidige model voor secundaire peritonitis voldoet aan de eisen voor wat betreft de vorming van abcessen, de overleving en de locale cytokinerespons. Het model is geschikt is om mechanismen te bestuderen met betrekking tot de vorming van intra-abdominale abcessen na chirurgische behandeling van gegeneraliseerde peritonitis.

In **hoofdstuk 3** wordt beschreven hoe het model zoals genoemd in het vorige hoofdstuk is toegepast om het effect te bestuderen van intra-abdominaal toegediende recombinant tPA (rtPA) op de vorming van abcessen. In eerdere experimentele studies is bewezen dat rtPA effectief is in het reduceren van abcessen in knaagdiermodellen voor peritonitis zonder dat er een toename van bloedingscomplicaties optreedt. Echter in deze studies was intra-abdominale behandeling met rtPA geassocieerd met een aanzienlijke en significant verhoogde mortaliteit, waarschijnlijk als gevolg van een verhoogde klaring van bacteriën uit de buikholte met als gevolg een bacteriëmie en sepsis, dan wel als gevolg van een "overdreven" locale immuunrespons. De huidige studie werd uitgevoerd om te bepalen of de intra-abdominale toediening van rtPA kan worden geoptimaliseerd door de manier van toediening en het doseringsschema aan te passen. Daarnaast werd onderzocht of het intraperitoneaal toedienen van rtPA geassocieerd is met een verhoogde inflammatoire respons in de buikholte, een verhoogde incidentie en ernst van bacteriëmie of toegenomen mortaliteit.
Vijfenvijftig ratten werden steeksproefsgewijs verdeeld in 3 groepen van 14 en 1 groep van 13. Bacteriële peritonitis werd geïnduceerd, gevolgd door chirurgische behandeling, zoals beschreven in hoofdstuk 2. Drie experimentele groepen ontvingen intraperitoneaal 1,25 mg rtPA 1 uur (rtPA1), 1 uur en 6 uur (rtPA2) of 1,6 en 24 uur (rtPA3) na inoculatie. De dieren in de vierde groep werden behandeld met een steriele zoutoplossing op alle genoemde tijdstippen. Er werden bloedmonsters afgenomen voor bacteriële kweken op 6 en 24 uur na inoculatie. Buikvochtmonsters werden afgenomen na 24 uur om spiegels van cytokines te bepalen en celtellingen uit te voeren. Na 5 dagen werden de ratten gedood door CO_2-asphyxie. De buik werd geïnspecteerd op de aanwezigheid en de locatie van abcessen.

Behandeling met rtPA leidde tot een significante reductie van abcesvorming. Ratten die 3 doses rtPA hadden ontvangen, vertoonden géén abcessen in tegenstelling tot 88% van de controles, en 58% en 33% van de ratten die respectievelijk 1 of 2 doses rtPA hadden ontvangen.
Sterfte, het verloop van het lichaamsgewicht en de incidentie van bacteriëmie werden niet beïnvloed door toediening van rtPA, evenmin als de celtellingen en de spiegels van TNF-α, IL-1β, IL-6 en IL-10. Er werden geen bloedingscomplicaties gezien.
De conclusie luidde dat rtPA de vorming van intra-abdominale abcessen vermindert zonder toename van mortaliteit en zonder de locale inflammatoire reactie te beïnvloeden.

Hoofdstuk 4 beschrijft een groter experiment waarin een vergelijking werd gemaakt tussen twee fibrinolytica die qua werkingsmechanisme verschillen. Opnieuw werd een peritonitis geïnduceerd in 80 mannelijke Wistar ratten, zoals eerder beschreven. De dieren werden verdeeld in 4 groepen van 20 ratten. Twee groepen werden behandeld met een intra-abdominaal fibrinolyticum. Een groep ontving 3 doses van 1.25 mg rtPA op 1, 6 en 24 uur na inductie van peritonitis, een tweede groep werd behandeld met 3 doses van 725.000 IU urokinase. Een controlegroep werd behandeld met een steriele zoutoplossing en een tweede controlegroep diende als negatieve eiwitcontrole en werd behandeld met 3 x 725.000 IU streptokinase. Van deze stof is bekend dat zij het stollingssysteem van de rat niet beïnvloedt. Er werden bloedmonsters genomen voor bacteriologische kweken op 6 en 24 uur na inductie van peritonitis en buikvochtmonsters op 24, 72 en 120 uren voor celtellingen en om spiegels van IL-6, IL-10 en TNF-α te bepalen. Na 5 dagen werden de dieren opgeofferd en de buikholte geïnspecteerd op de aanwezigheid en locatie van abcessen.
RtPA en urokinase bleken beiden duidelijk significant het aantal abcessen te reduceren zonder dat er negatieve bijwerkingen waren. Er werden geen bloedingscomplicaties gezien.
In het algemeen gesteld leidde fibrinolytische therapie tot een verminderd aantal intra-abdominale neutrofielen en lymfocyten en tot een verhoging van het aantal macrofagen en eosinofielen gedurende het experiment. Echter het beloop van de IL-6 en IL-10 (afname gedurende het experiment) of TNF-α (toename gedurende het experiment) spiegels in het buikvocht werd niet beïnvloed door rtPA.
Als conclusie werd gesteld dat in een rattenmodel voor de behandeling van secundaire peritonitis met zowel rtPA als urokinase effectief en veilig abcesvorming kon worden tegengegaan.

In de klinische praktijk zal de aanvang van adequate therapie variëren als gevolg van de tijd die verloopt tussen de aanvang van ziekte en presentatie van klinische symptomen of als gevolg van het niet direct stellen van de juiste diagnose. Hierbij komt dat tot op heden de (experimentele) behandeling met rtPA alleen gedurende de eerste 24 uur na inductie van peritonitis is toegepast.
Om deze reden beschrijft **hoofdstuk 5** het effect van uitgestelde en verlengde behandeling van het intraperitoneaal toegediende rtPA. Vooruitkijkend naar een eventuele noodzaak om het risico van deze behandeling nog verder te reduceren werd ook onderzocht wat het effect was van een dosisverlaging.
Bacteriële peritonitis werd geïnduceerd in mannelijke Wistar ratten zoals eerder beschreven. Chirurgische interventie werd na één uur uitgevoerd. Er werden buikvochtmonsters genomen op 24 en 72 uur na inductie van peritonitis om IL-6, IL-10 en TNF-α spiegels te meten en celtellingen uit te voeren. Na vijf dagen werd de buikholte geïnspecteerd op de aanwezigheid van abcessen.
Er werden twee experimenten uitgevoerd. In experiment I werden vier groepen ratten behandeld met rtPA. Een groep werd behandeld met 1,25 mg rtPA na 1, 6 en 24 uur (rtPA), een andere groep ontving een verlengde behandeling: 7 doses werden toegediend gedurende 72 uur (rtPA prol). Een groep ontving 3 doses van rtPA na een uitstel van 24 uur (rtPA 24) en een andere groep na een uitstel van 48 uur (rtPA 48). Een controlegroep werd behandeld met een steriele zoutoplossing op 1, 6 en 24 uur. In het tweede experiment werden twee groepen behandeld met respectievelijk 3 x 0,25 mg of 1,25 mg rtPA (gedurende de eerste 24 uur).
Vroege toediening van rtPA in beide doses leidde tot een significante reductie van het aantal ratten met abcessen alsmede het aantal abcessen per rat. Uitgestelde behandeling leidde nog steeds tot een significante daling van het aantal abcessen per rat, maar niet tot daling van de incidentie van abcessen. Er werden geen negatieve bijwerkingen van de intra-abdominale rtPA behandeling gezien. Er waren geen verschillen van betekenis voor wat betreft de locale inflammatoire respons. RtPA was het meeste effectief bij continuering van de toediening gedurende 72 uur. Echter, er werd een verhoogde mortaliteit waargenomen na verlengde behandeling met de standaard dosis van 1,25 mg. Hiervoor was geen duidelijke verklaring.
Deze resultaten bevestigden de potentiële waarde van rtPA als toevoeging aan de standaardbehandeling van peritonitis in de klinische situatie. De veiligheid van doseringsschema's dient te worden getest in een fase II klinisch onderzoek. Het effect van een verlengde behandeling met lagere doses rtPA dient onderzocht te worden in die gevallen waarin langdurige behandeling geïndiceerd is.

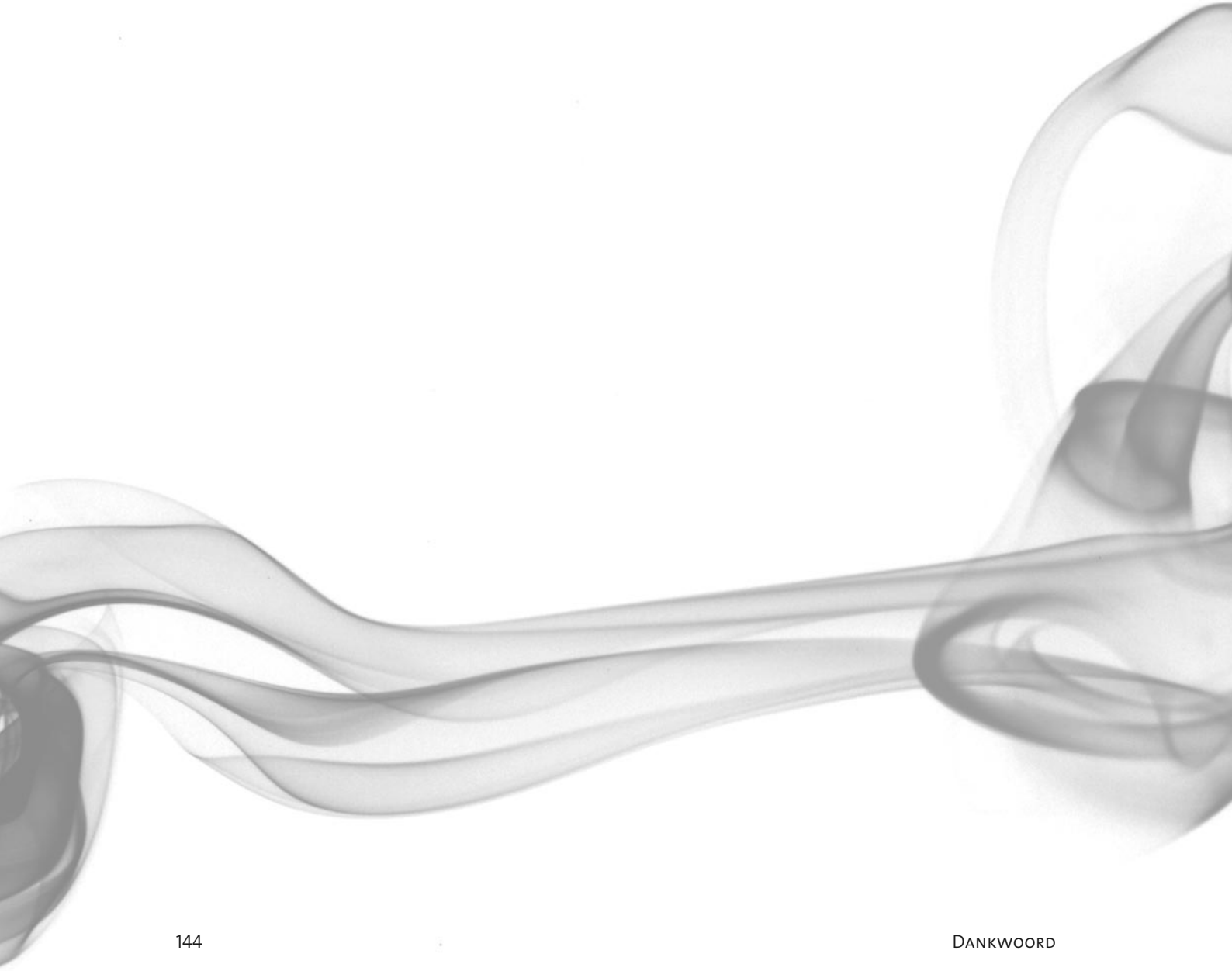
Het toedienen van systemische antibiotica is een belangrijk element van de behandeling van peritonitis. Tot nog toe was er nog geen antibiotische therapie toegepast in de hiervoor beschreven studies studies. Dit om specifiek het effect van het intra-abdominaal toedienen van fibrinolytica te bestuderen.

Hoofdstuk 6 beschrijft het effect van de systemische toediening van antibiotica op intra-abdominale abcesvorming, zowel met als zonder intraperitoneale toediening van rtPA.
Tweeënzeventig ratten werden door loting verdeeld in 4 gelijke groepen. In alle ratten werd de peritonitis opgewekt als eerder beschreven. De eerste (controle) groep kreeg geen verdere medicamenteuze behandeling, de tweede groep (rtPA) werd behandeld met 1,25 mg rtPA intra-abdominaal, de derde groep werd behandeld met systemische antibiotica (AB) en de vierde groep met systemische antibiotica en intra-abdominaal toegediende rtPA (rtPA + AB groep). Antibiotische therapie werd gedurende 3 dagen toegediend en bestond uit 15 mg/hg Metronidazol en 15 mg/kg Ceftriaxon, tweemaal daags. Er werden bloedmonsters afgenomen voor bacteriologische kweken op 24 uur na inductie van peritonitis. Buikvochtmonsters werden afgenomen op 24, 72 en 120 uur en werden gebruikt voor celtellingen en om spiegels van IL-6, IL-10, TNF-α te bepalen. Zoals in de eerdere experimenten werden de dieren opgeofferd na 5 dagen en werd de buikholte geïnspecteerd op de aanwezigheid en de locatie van abcessen.
Antibiotica op zich hadden geen invloed op de vorming van abcessen, maar intra-abdominale toediening van rtPA leidde tot een significante reductie van het aantal ratten met abcessen alsook de abceslast (als maat voor de totale hoeveelheid abcessen) per rat. Dit bleek ook het geval te zijn in de ratten die naast rtPA eveneens behandeld werden met systemische antibioticatherapie.
Eén rat overleed voor het einde van het experiment. Er werden geen negatieve neveneffecten gevonden en er waren geen verschillen van betekenis voor wat betreft de locale inflammatoire respons. De conclusie luidde dat intra-abdominale toediening van rtPA leidt tot een consistente afname van abcessen. De systemisch toediening van antibiotica beïnvloedt abcesvorming niet. Naast antibiotica is rtPA is een waardevolle aanvulling bij de behandeling van peritonitis.

Vaak wordt als deel van de chirurgische behandeling van peritonitis een intestinale anastomose geconstrueerd of herstel van een visceraal orgaan uitgevoerd. Zoals aangetoond in de eerder uitgevoerde experimenten leidt fibrinolytische therapie tot afname van het aantal intra-abdominale

abcessen. Van de andere kant is fibrinevorming een essentieel proces tijdens vroege wondgenezing welke moet leiden tot een provisorische matrix voor de initiële wondstabiliteit en celmigratie. Hypothetisch zou reductie van fibrinevorming dus kunnen leiden tot verstoringen van de wondgenezing, in dit geval in het bijzonder tot verstoring van de genezing van een anastomose.

In **hoofdstuk 7** worden de mogelijke effecten van de intra-abdominale toediening van rtPA op naadgenezing en genezing van de fascie van de buikwand beschreven na chirurgische behandeling voor peritonitis in een rattenmodel.
Peritonitis werd geïnduceerd en behandeld in 148 ratten zoals hierboven beschreven. Er werden twee experimenten uitgevoerd. In experiment I kreeg een groep op 1, 6 en 24 uur na inductie van peritonitis 1,25 mg rtPA intraperitoneaal toegediend. Een tweede groep werd op dezelfde wijze behandeld, echter met een dosis van 0,25 mg. Een derde groep werd behandeld met driemaal 1,25 mg rtPA beginnend na 24 uur. De vierde groep was de controlegroep en werd behandeld met een steriele zoutoplossing op 1, 6 en 24 uur. Elke groep bestond uit 30 ratten. In elke groep werd, na chirurgisch debridement 1 uur na injectie van het bacteriële inocilum, 2 cm van het colon descendens gereseceerd op 3 cm proximaal van de peritoneale omslagplooi. Continuïteit werd hersteld door middel van een end-to-end anastomose met 8 volledige wanddikte omvattende, inverterende, staande hechtingen. In elke groep werd de helft van de dieren gedood op de derde dag en de andere helft op de zevende dag na de operatie. In experiment II werd een ileumresectie en anastomose uitgevoerd direct na chirurgisch debridement van de buikholte. De dieren werden verdeeld in twee groepen (n=14), een rtPA groep en een controlegroep. Alle ratten werden op de derde postoperatieve dag gedood. In beide experimenten werden de abcessen geteld, barstdruk, breeksterkte en het hydroxyprolinegehalte van anastomoses en van de abdominale fascie gemeten.
Behandeling met rtPA in beide doses leidde tot een significante reductie van abcessen. De sterkte van zowel colonnaden als de wonden van de buikwandfascie nam significant toe van dag 3 tot dag 7. Er waren geen significante verschillen tussen de groepen met betrekking tot barstdruk, breeksterkte en hydroxyprolinegehalte. Dit gold zowel voor de colon- als voor de dunne darm anastomoses.
De conclusie luidde dat toediening van rtPA leidt tot een consistente reductie van abcessen en geen invloed heeft op de genezing van intestinale anastomoses of de buikwandfascie.



Dankwoord

DANKWOORD

Geachte promotor, beste Rob,
Ooit heb je mij uit het hoge noorden weggelokt om in Nijmegen te komen werken. De aanleiding lag ongetwijfeld in een gedeelde belangstelling voor peritonitis en andere chirurgische infecties. Je bleek niet alleen een enthousiaste onderzoeker, maar ook een uitstekend chirurg en gewaardeerde collega. Je hebt jouw rol als promotor met verve vervuld. Uit een soms niet te stuiten stroom van ideeën en gedachten is uiteindelijk de definitieve vorm van dit proefschrift ontstaan. Dank voor al hetgeen jij bij hebt gedragen.

Geachte copromotor, beste Harry,
Ooit probeerde jij mij te interesseren voor het schrijven van een proefschrift. De letterlijke woorden waren: "Je kunt hier zo promoveren. Ik heb nog genoeg op de plank liggen. Je hoeft het alleen maar op te schrijven." Gelukkig was de werkelijkheid anders. Volgens mij ligt jouw plank voller dan ooit en ben ik niet diegene die hem leger zal maken. Mede door jouw innovatieve ideeën, jouw creatief denken en nooit aflatende kritische blik op opzet, uitvoering en uitwerking is dit proefschrift geworden tot wat het is. Zeer veel dank voor jouw bijdrage aan dit proefschrift.

Geachte copromotor, beste Thijs,
Gaande de totstandkoming van dit proefschrift werd mij al vlot een aantal zaken duidelijk. Als jij "ja" tegen een klus zegt, dan maak jij die "ja" volledig waar. Je bent een zeer kritische en toegewijde begeleider die uitstekend kan schrijven, corrigeren en redigeren. Ik heb bijzonder veel van je geleerd en heb je leren waarderen als een uiterst verstandig, consequent, betrouwbaar en aangenaam mens. Op gevaar van dat je mij eraan gaat houden: ik zou het zo nog een keer doen (are you kidding me!!!).

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De manuscriptcommissie,
Ik dank de manuscriptcommissie voor het zeer kritisch lezen van het manuscript.

Collega stafchirurgen, fellows, chivo's, arts-assistenten en secretariaat van de afdeling Heelkunde,
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Naast een meer dan full time baan als secretaresse voor een aantal collega's zag jij nog kans om mij te helpen met allerlei hand-en-spandiensten rondom dit proefschrift. Klein maar dapper, zou ik zeggen. Zeer veel dank.

Dr O.H Buyne, beste Otmar Sr.
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So so lobi.

De paranimfen,
Beste Peter, wij kennen elkaar relatief kort, maar al snel werd mij duidelijk dat je als gewaardeerde collega, als prettig gestoord mens maar ook als on-the-spot organisator vele talenten bezit. Na jouw optreden als presentator van een Karaoke show was ik geheel overtuigd.
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Mijn dames: Loïs, Nicchelle en Caïsa.
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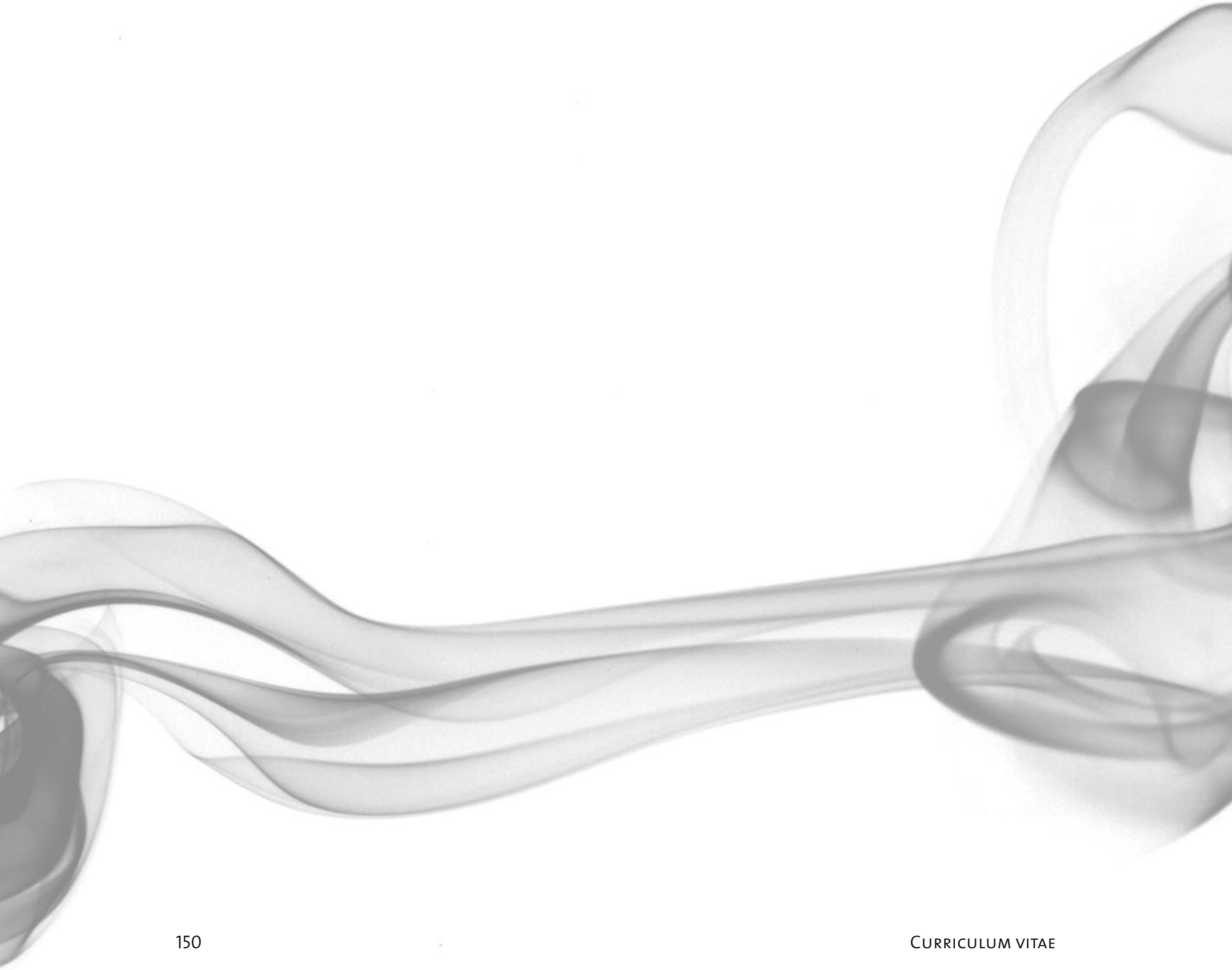

Curriculum Vitae

CURRICULUM VITAE

Otmar Buyne werd in 1965 geboren in Utrecht.
Na enige jaren heen en weer te hebben gereisd tussen Curaçao, Suriname en Nederland deed hij in 1982 eindexamen op het Maartenscollege in Haren (Groningen).
Na een jaar Farmacie werd hij ingeloot voor de studie Geneeskunde in Groningen. Hij behaalde zijn doctoraal examen in 1991. Tijdens het volgen van de co-schappen in het St Elisabeth Hospital in Willemstad, Curaçao, werd de belangstelling voor de heelkunde geboren. Na terugkeer in Nederland volgde hij een keuze co-schap chirurgie in het Medisch Centrum Leeuwarden, toen nog met dr. P. de Vogel als opleider.
Na een periode van drie jaar, waarin hij keuringsarts, arts-assistent Intensive care gecombineerd met heelkunde en ook arts op het Brandwondencentrum Groningen was, werd hij aangenomen voor de opleiding heelkunde.
Het perifere deel van de opleiding werd gevolgd in het Martini Ziekenhuis Groningen met als opleider dr. L. Vos en later dr. H. Oeseburg. De laatste drie jaren van de opleiding heelkunde werden onder leiding van prof. R van Schilfgaarde gevolgd in het Academisch ziekenhuis Groningen nu UMCG geheten.
Na een korte periode van waarneming in het Wilhelmina Ziekenhuis te Assen heeft hij bijna een jaar als tijdelijk staflid Traumatologie in het UMCG gewerkt onder leiding van prof HJ. ten Duis. Hierna werd hij aangesteld als fellow gastrointestinale chirurgie en traumatologie in het Universitair Medisch Centrum Nijmegen St Radboud, waar hij uiteindelijk toe is getreden tot de staf Gastrointestinale chirurgie en tot op heden werkzaam is.
Hij woont in Nijmegen samen met Anita Samsom en hun drie dochters Loïs, Nicchelle en Caïsa.